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**Analysis of *cis*-Regulatory Elements
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Expression of the Rice Seed Storage Protein
Gene, *GluA-3***

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1997

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Abbreviations

bp	base pair(s)
CaMV 35S	Cauliflower mosaic virus 35S RNA
DAP	day(s) after pollination
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
E	Einstein
FW	fresh weight
GUS	β -glucuronidase
LUC	firefly luciferase
h	hour(s)
kb	kilo base(s)
min	minute(s)
4-MU	4-methyl umbelliferone
4-MUG	4-methyl-umbelliferyl β -D-glucuronide
NOS	nopal synthase
P	promoter
pA	poly A tail
PCR	polymerase chain reaction
RNA	ribonucleic acid
sec	second
Tris	tris(hydroxymethyl)aminomethane
X-gluc	5-bromo-4-chloro-3-indoyl β -D-glucuronide

CHAPTER I

General Introduction

Plants store a significant amount of their nitrogen, sulfur, and carbon reserves as storage proteins in seed tissue, which are utilized by the seedling for growth and development. Based on their solubility properties, these storage proteins are divided into four classes: albumins, globlins, prolamins and glutelins (Shotwell and Larkins, 1989). The proportion of those proteins in seeds are different among the plant species: globlins, chracterized by their solubility in saline solutions, serve as the major nutrient reserves in the embryonic tissues of both dicot and monocot seed, whereas the alcohol-soluble prolamins and acids- or alkaline- soluble glutelins are accumulated in the endosperm tissue of monocots (Shotwell and Larkins, 1989; Shewry and Tatham, 1990).

The expression of seed storage protein genes is strictly regulated in the spatial- and temporal- specific manner, thus provides the model system for studying the regulation of plant genes (Shotwell and Larkins, 1991). Up to date, a large number of seed storage protein genes have been isolated (maize zein; Pedersen *et al.*, 1981: pea legumin; Lycett *et al.*, 1984: soybean glycinin; Macro *et al.*, 1984: French bean phaseolin; Slightom *et al.*, 1983: wheat glutenin; Thompson *et al.*, 1985: barley hordein; Cameron-Mills and Brandt, 1988: rice glutelin; Takaiwa *et al.*, 1987a). Some *cis*-regulatory regions and motifs (cf. G-box motifs, RY motifs and endosperm motifs) of these genes responsible for seed- or the developmental stage- specific expression have also been reported (Thomas, 1993). Most of these genes were identified because of

their phylogenetic conservation, although some motifs (cf. RY motif and endosperm motif) have been studied. However, a number of detailed studies have failed to provide a definitive picture of the roles of specific sequences in the gene expression. Thomas (1993) suggested two possibilities to this conundrum. First, regulatory ensembles of seed protein genes are extensive, often including more than a kilo base of upstream sequence. Second, seed protein gene regulatory ensembles are the result of combinatorial interactions of multiple DNA elements.

Glutelin accounts for up to 80% of total endosperm protein of rice (*Oryza sativa* L.: Yamagata *et al.*, 1982). The glutelin is encoded by a small multigene family of about 10 copies per a haploid genome. Eight genes have already been sequenced at both the cDNA and genomic DNA levels (Takaiwa *et al.* 1987a, b, Okita *et al.* 1989, Takaiwa *et al.* 1989, 1991a, c). All the glutelin genes are specifically expressed in the maturing seed (Okita *et al.* 1989, Takaiwa *et al.* 1991a, b). Although it is considered that these genes are controlled by common regulatory mechanisms, there is little sequence homology among the genes other than a few conserved sequences, such as the AACAA (AACAACTCTATC), GCN4 (ATGAG/CTCAT) and GCAA (G/CCAAAATGA) motifs (Takaiwa *et al.* 1991c, 1996). It has been shown that these motifs are involved in the endosperm specific expression (Takaiwa *et al.* 1991b, Zheng *et al.* 1993, Zhao *et al.* 1994, Takaiwa *et al.* 1996). In addition, it was also demonstrated that these motifs are recognized by nuclear proteins (Kim and Wu 1990). However, further studies are required to understand the common regulatory mechanisms underlying the spatial-specific expression of the glutelin genes. Especially, the proximal region of the gene has not been well analyzed, although the AACAA and the

GCN4 motifs are conserved between position -100 and -60 in all the members of glutelin gene. Furthermore, in view of the fact that *cis*-regulatory elements of many specific genes are arranged in modules of short DNA segments (Benfey and Chua, 1990; Shen and Ho, 1995), specific expression of seed storage protein genes may also depend on combinatorial arrangements of distinct regulatory modules.

In this thesis, to obtain the fundamental evidences for improving the rice grain quality by genetic engineering and for studying the regulatory mechanism of seed storage protein genes, the author analyzed the *cis*-regulatory region of the rice seed storage protein gene, *GluA-3*, that is differently expressed in temporal specific manner from the other glutelin genes, using transgenic tobacco system. The results clearly showed the regulatory elements of *GluA-3* responsible for the endosperm-specific expression. It was also characterized the combinatorial regulation of the elements and the common regulatory mechanism(s) of the glutelin genes.

In Chapter II, the *cis*-region of *GluA-3* required for the endosperm-specific expression and the quantitative control was characterized by both the 5' deletion analysis and the gain-of-function experiments.

Chapter III denotes the developmental expression of the restricted *GluA-3* promoter (-897/+11~-437/+11) in tobacco by the double transformation analysis.

In Chapter IV, roles of two motifs (AACA motif, -77/-63 and GCN4 motif, -102/-94) in the proximal region of *GluA-3* were characterized by the site-specific mutagenesis of these motifs.

The author assessed the common regulatory elements required for the endosperm-specific expression of the glutelin genes

by the chimeric promoters consisting of the *GluA-3* and the *GluB-1* genes, and defined the combinatorial expression of the elements and the common regulatory mechanism(s) among the glutelin genes in Chapter V.

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CHAPTER II

Identification of the *cis*-region of *GluA-3* by the 5' deletions and the gain-of-function analyses

Glutelin is the major seed protein of rice (*Oryza sativa* L.) and constitutes about 80 % of the total starchy endosperm protein (Yamagata *et al.*, 1982). The biochemical characteristics of glutelin are very similar to that of leguminous 11S and 12S globulins (Zhao *et al.*, 1983; Wen and Luthe, 1985) and the oat 12S globulin (Robert *et al.*, 1985; Schotwell *et al.*, 1988). As shown in many seed storage protein genes, glutelin is also encoded by a small multigene family of about 10 copies per haploid genome. Eight genes have already been sequenced at both the cDNA and genomic DNA levels. These genes are divided into two subfamilies (*GluA* and *GluB*) based on their sequence homologies, and each subfamily includes four or five members (Takaiwa *et al.*, 1987a; Takaiwa *et al.*, 1987b; Masumura *et al.*, 1989; Okita *et al.*, 1989; Takaiwa *et al.*, 1989; Takaiwa *et al.*, 1991a). The degree of homology between the *GluA* and *GluB* members is 60 - 65 %, whereas members within the same subfamily share more than 80 % homology. Takaiwa and Oono (1991c) indicated that the distinction of the glutelin subfamilies may have occurred at almost the same time as the speciation of rice and oats. A few conserved sequences are found out in the 5' flanking regions of glutelin genes. Those are named AACA, GCN4 and GCAA motifs, and considered as candidates for the *cis*-regulatory elements of the temporal and spatial specific expressions of glutelin genes (Takaiwa *et al.*, 1991a, 1996).

GluA-3, which belongs to the *GluA* subfamily (Takaiwa and

Oono, 1991c), is an unique gene in the respect of the nucleotide sequence homology and the temporal expression manner among the *GluA* subfamily. The 5' flanking regions of *GluA-1* and *GluA-2* share about 95 % nucleotide sequence homology, whereas those of *GluA-3* share only about 80 % homology between those of *GluA-1* and *GluA-2* (Okita *et al.*, 1989; Takaiwa and Oono, 1991c). No significant homology is observed in further upstream regions from -186 bp, between *GluA-3* and *GluA-1* or *GluA-2* (between Gt3 and Gt2 or Gt1 (Okita *et al.*, 1989). Furthermore, the *GluA-3* gene includes less number of the conserved motifs than the other glutelin genes: i. e., more simple interaction between the motifs would lead to more clear analytic results.

In this chapter, the 897 bp 5' flanking regions of the *GluA-3* were analyzed to identify *cis*-regulatory elements responsible for developmental control by deletion mutation from the 5' end and gain-of-function experiments using CaMV 35S truncated promoter (-90/+8). The results showed that at least two spatially separated elements required for the seed specific expression is located between -437 and -317 and consists of a general enhancer element and a unique negative element, individually.

Materials and Methods

Construction of GluA-3 5' deletion mutants

A 2.8 kb *GluA-3* genomic clone (pREX21) from rice mature seeds (*Oryza sativa* L. cv. Mangetsu-mochi) was isolated by Takaiwa and Oono (1991c). This genomic clone contains a 0.9 kb

5' flanking region, a 1.8 kb coding region and a 0.3 kb 3' flanking region. The 0.9 kb *Xba* I/ *Bam*H I fragment from -897 to +11 bp, relative to transcription initiation site, was amplified from the genomic clone by the PCR method (PCR kit, Promega Co. Ltd., USA), and subcloned into pBluscript SK+. After cutting at the unique *Pst* I and *Spe* I sites of the plasmids, a series of *Exo* III nuclease deletion mutants of the 0.9 kb fragment was created and the extent of deletion was sequenced by dideoxy chain termination method (Sanger *et al.*, 1977). Ten deletion mutants were isolated and cloned into the *Hind* III and *Bam*H I sites on the binary vector pGPTVL-bar to create the chimeric constructs with a firefly luciferase (LUC) gene as shown in Figure II-1. The pGPTVL-bar is a reconstructed binary vector of pGPTV-bar (Becker *et al.*, 1992) by conversion of the β -glucuronidase gene (GUS) to LUC as the reporter gene at the same restriction enzyme sites.

***Construction of three chimeric promoters consisting of
GluA-3 putative enhancer and CaMV 35S promoter truncated
region***

The full length of the putative positive sequence (DA1; -439/-316), upstream half of the sequence (DA2; -439/-378) and downstream half of the sequence (DA3; -385/-316), were amplified by the PCR method from the pREX21 using synthetic primers containing *Hind* III and *Bam*H I restriction sites at the 5' and 3' ends, respectively. These amplified sequences were cloned into the upstream region of the CaMV 35S truncated promoter (-90/+9) in pLP19 binary vector which has the GUS reporter gene (Szabados *et al.*, 1990) as shown in Figure II-2.

Plant transformation and cultivation

The plasmids pGPTVL-bar and pLP19 containing *GluA-3* genes were introduced into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating using pRK2013 as helper. Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) was used for the *Agrobacterium* infection. The infected tobacco leaf disks were cultured and regenerated as described by Takaiwa *et al.* (1991b). The transformants were selected by bialaphos (10 μ M) or Kanamycin (100 mg/L), and confirmed by Southern hybridization (Maniatis *et al.*, 1982) and PCR method. After regeneration, all the transformants were transferred into nutrient soil and grown in a greenhouse.

LUC and GUS assays

One pod from each of 7 to 20 transformed tobacco plants was harvested at every 4 or 5 days between 7 to 24 days after pollination (DAP). To compare the maximum activity in the seeds for each construction, the highest value during the endosperm development was used. Pods were frozen instantly in liquid nitrogen and stored at -80 °C until use. A leaf disc and a section of stem and root were also harvested and stored similarly. Seeds from a quarter of the pod and about 0.1g fresh weight (FW) of the other parts were collected and homogenized in liquid nitrogen, and mixed with 300 μ l of LUC or GUS extraction buffer. The homogenate was centrifuged and the supernatant used for LUC or GUS assay, and total soluble protein assay. LUC assays were performed essentially as described by Millar *et al.* (1992) with a luciferase assay kit supplied

by Toyo Ink Co. LUC fluorescent intensity was measured as photon count by a luminometer (Lumicounter ATP-300, Advantec, Tokyo, Japan) for the first ten seconds after the addition of the luciferin. GUS assays were performed as described by Jefferson *et al.* (1987). GUS fluorescence was measured using a spectrofluorometer (model RF540, Shimadzu Co.Ltd., Kyoto, Japan) calibrated against 4-MU.

Total protein content was determined by the method of Bradford (1976) with a kit supplied by Bio-Rad laboratories. For histochemical and fluorometrical staining of LUC activity, hand-cut thin section of the pod including seeds and half cut seed was dipped into luciferin solution for a few minutes at room temperature or in X-glucuronide solution for over night at 37 °C, respectively. The sectioned seed was observed in a dark room with high sensitivity CCD microscope and analyzed by image processing and measuring systems (ARGUS - 50/MP HAMAMATSU Photonics K.K., Hamamatsu, Japan). And, in the case of GUS activity, the sectioned seed was observed under a stereoscopic microscope.

Data analysis

LUC activity was rated as photon count per second per microgram of the total soluble protein. GUS activity was also normalized by a microgram of the total soluble protein. Non-transformed plants were used as a negative control. LUC and GUS activities for each plant were statistically analyzed by Duncans' multiple-range test or t-test.

Results

The full length (925 bp; -897/+11 from the transcription start site; d897) in the 5' flanking region directed high LUC activity in developing seed of the transformed tobacco (Figure II-3). While the deletion construct d839 showed a slightly decreased activity, five constructs from d760 to d437 showed almost the same or rather increased LUC activities with the deletions compared to d897. In contrast, a further deletion to position -317 (d317) resulted in a remarkable reduction in the activity. More deletions, to position -138 (d138), did not affect the low activity. The average LUC activity in each of three constructs from d317 to d138 were about 10% of the d897 activity. However, the low activities observed in these constructs were still above the background. These results indicate that the sequence -437/-317 is important for the positive quantitative regulation of *GluA-3* gene.

On the other hand, the leaf, shoot and roots showed comparative little or no LUC activity in every 5' deletion constructs (Table II-1). Even if the scarcely higher LUC activity than the background level was shown in leaf of transformants harboring the d897 to d437 constructs, the differences disappeared with the further deletions to d317 and more. Thus, the transformants harboring the d897 to d437 constructs indicated definitive seed specific expression, where the LUC activities in seed were 7 to 15 times higher than that in the other tissues. However, respective of the remarkable decreasing of the activity in seed, the spatial specificity was hard to detected in the constructs d317, d221 and d138.

In addition, the enhancement effect of the sequence -437/-317 was examined by the gain-of-functional experiment (Figure II-4). The full length of the putative positive sequence (DA1; -439/-316), upstream half of the sequence (DA2; -439/-378), and downstream half of the sequence (DA3; -385/-316) were fused to the CaMV 35S truncated promoter (-90/+9), individually, and their promoter activities were analyzed as the GUS activities in transgenic tobacco. The DA1 and DA3 sequences enhanced the promoter activity in seed by about 10-fold compared to that of the CaMV 35S truncated promoter, however, the DA2 sequence could not enhance it. The activity of the DA1 and DA3 sequences fused to the CaMV 35S truncated promoter (-90/+9) were much higher than that fused to the native *GluA-3* truncated promoter (see Chapter V).

While the levels of GUS activity enhanced by the DA1 and DA3 sequences was almost the same in seeds, the enhancement effect of the DA3 sequence was three to five times higher than that of the DA1 sequence in the other tissues. In other words, the levels of GUS activities enhanced by the DA3 sequence were quite high in leaf, stem and root (Figure II-4), but that enhanced by the DA1 sequence were not. These results indicate that the DA1 sequence might consist of two functionally different motifs. Thus, DA3 sequence includes a general positive element and the DA2 sequence includes a specific negative element for masking the *GluA-3* gene expression other than the seed.

Furthermore, the histochemical and fluorometrical analyses indicated the more detailed information about the spatial specificity: the LUC fluorescence were distinguishably stronger in the endosperm than in the embryo of the transformant containing the

constructs through d897 to d437, whereas that were considerably weak and almost the same in both tissues of the transformants containing constructs through d317 to d138 (Fig. II-5). These results indicated that the spatial-expression regulation of the *GluA-3* promoter in tobacco was similar to that in the mother plant, and the down stream sequence at least from position -437 contains the endosperm specific expression. In addition, while the DA1 sequence induced the endosperm specific GUS staining, the DA3 sequence showed the high GUS staining in the entire seed tissues including the embryo (Fig. II-6). The DA2 sequence induced little or no GUS staining in both tissues. Taken altogether, the combination of the two sequences, DA2 and DA3, was responsible for the visible endosperm-specific expression.

DISCUSSION

The progressive deletion of the 5' flanking region of *GluA-3* induced an one-stepped depression in the LUC activity (Fig. II-2). This suggests a positive quantitative control element exists in a narrow region of the sequence between position -437 and -317 from the transcriptional start site. In addition, histochemical and fluorometrical analyses showed that the *cis*-regulatory element(s) responsible for endosperm-specific expression of the *GluA-3* gene resides within the -437/-317 sequence and/or the downstream region. The one-stepped depression of the reporter activity induced by the 5' deletions has also been shown in the other glutelin gene, *GluA-2* (Takaiwa *et al.*, 1991b, Zheng *et al.*, 1993), in which the similar positioned sequence (-441/-237) confers the quantitative regulation.

Some conserved motifs in glutelin genes and the other genes, AACA motif (AACAAACTGCATT; -433/-421), GCAA motif (CCAAAATGT; -355/-337), G-box-like motif (TGACGTGG; -386/-379) and endosperm-like motif (TTTAAAGT; -325/-318) could be identified in the positive regulatory regions of *GluA-3*. Especially, the AACA and GCAA motifs are critically conserved at the relative same positions in all members of the rice glutelin gene family (Takaiwa *et al.*, 1996). The G-box-like motif (CACGTG) is considered as a binding site for DNA binding proteins basic leucine zipper (bZIP). One of the DNA binding proteins, the rice transcriptional activator-1 (RITA-1), is also able to bind the G-box motifs (Izawa *et al.* 1994). Another candidate, the endosperm motif is conserved in many cereal storage protein genes (Forde, *et al.*, 1985). Quayle and Feix (1992) observed that a 43 bp sequence containing the endosperm motif of 19 kDa zein promoter activated the expression of the core promoter of CaMV 35S gene in maize endosperm protoplasts.

On the other hand, Zhao *et al.* (1994) have recently shown the existence of positive elements between position -955 (-945) and -736 (-726) and between position -350 (-346) and -266 (-263) by the 5' deletion analysis using Gt3 (*GluA-3*) gene (Numbers in parenthesis are base-pairs relative to the transcriptional start site of Gt3 as indicated by Zhao *et al.*, 1994). Since the author analyze the 5' flanking region of the *GluA-3* gene between -897 and +11, the existing of the additional positive element in the far upstream region of -955/-898. It is suggested that the region between positions -350 and -317 includes the quantitative regulatory element of the *GluA-3* gene expression.

Subsequently, the author constructed three types of chimeric

promoters which were composed of a part of the positive regulatory regions of *GluA-3* (DA1, -439/-316; DA2, -439/-378; DA3, -385/-316) and the CaMV 35S truncated promoter (-90/+9). The results of gain-of-functional experiment clearly showed the positive regulatory function of the region between position -385 and -316 of *GluA-3* (DA3; Figure II-2). When the region was fused to the upstream of the CaMV 35S truncated promoter (-90/+9), a high GUS activity was observed not only in the seed, but also in the other tissues. The endosperm-like motif, the GCAA motif, and a part of the G-box-like motif are included in this region. Besides the general positive element (DA3), the adjacent 61 bp upstream region (DA2, -439/-378) was also suggested to be involved in the endosperm specific expression of *GluA-3* by the gain-of-functional experiment. This region could not activate the level of the transcription, but specifically silenced the activity by the DA3 sequence in the tissues other than seed. Such a negative *cis*-elements were demonstrated in several reports (Bustos *et al.*, 1991; Burow *et al.*, 1992; Leyva *et al.*, 1992). However, the negative element found in the rice glutelin gene is unique in that it contributes only in the spatial regulation. As the candidate for the negative element, the AACA motif and the G-box-like motif were identified in the 61 bp upstream region.

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Summary

Ten deletion mutants of the 897 bp 5' flanking regions of the rice storage protein glutelin gene (*GluA-3*) were constructed from the 5' end to identify *cis*-regulatory elements responsible for endosperm-specific expression. These were transcriptionally fused to the firefly luciferase reporter gene (LUC) and introduced into tobacco (*Nicotiana tabacum* cv. Petit Havana SR1). In addition, the author also constructed chimeric genes consisting of the putative positive element of *GluA-3* suggested by the 5' deletion analysis, CaMV 35S truncated promoter region (-90/+8) and GUS reporter gene, to clarify the enhancement effect.

Results indicated that a sequence between -437 and -317, from the transcriptional start site, is important for the positive quantitative regulation of the *GluA-3* gene expression. Removal of this sequence resulted in drastically decreasing the promoter activity. Further gain-of-function experiments resulted that the 61 bp downstream half of the sequence (-378/-317) acted as a general positive element, since the sequence increased the promoter activity in every tissue analyzed. However, the positive regulatory sequence with an adjacent upstream of 61 bp sequence (-439/-378) governed endosperm specific expression. Therefore, the additional 61 bp sequence would include a specific negative element depressing the expression in all tissues other than endosperm.

CHAPTER III

The double transformation analysis for the developmental expression

Accumulation of seed storage proteins is under strict developmental control and provides an excellent system to study the molecular mechanism of spatial and temporal gene regulation (Zheng *et al.*, 1993; Müller *et al.*, 1995; Wobus *et al.*, 1995). To date, some of them, e.g. a French bean β -phaseolin gene (Sengupta-Gopalan *et al.*, 1985), wheat low molecular glutenin genes (Colot *et al.*, 1987), a barley B hordein gene (Marris *et al.*, 1988) and a zein gene from maize (Schernthaner *et al.*, 1988), have been characterized in terms of developmentally regulated expression.

The expression of the rice glutelin gene is considered to be controlled under either the transcriptional levels (Okita *et al.*, 1989; Takaiwa and Oono, 1991c) and post-transcriptional levels (Kim *et al.*, 1993). The mRNA level of *GluA-3* increases rapidly after anthesis and reaches the maximum level at 10 days after flowering (DAF) and then drops off, while those of the other glutelin genes are first detected at 6 DAF and then reach the plateau at 14 to 20 DAF. To date, the temporal expressions of the reporter gene, which individually controlled by *GluA-2* (Takaiwa *et al.*, 1991b) and *GluA-3* (Leisy *et al.*, 1989) have been observed in transgenic tobacco plants. The loss-of-function experiments recently indicated that the upstream of the promoter sequence of -945/-726 is required for the temporal specific expression of the *GluA-3* gene (Zhao *et al.*, 1994).

In this chapter, the author attempted whether the *cis*-regulatory element(s) responsible for the specific early temporal

expression of the *GluA-3* gene resides within the position -897 to +11 of *GluA-3*. The double transformation analysis, in which the two different constructs of *GluA-3*/LUC and *GluA-2*/GUS or *GluB-2*/GUS are transferred into single explant, were used to demonstrate the difference between the regulation of two promoters, *GluA-3* and *GluA-2* or *GluB-2*.

Materials and Methods

The double transformation of the 5' deleted promoters of *GluA-3* and *GluA-2* or *GluB-2* promoter

Prior to the transformation of a series of the *GluA-3* :: LUC construct, plasmids containing the other constructs of *GluA-2* (-1329/+1) :: GUS (Takaiwa *et al.*, 1991b) or *GluB-2* (-1209/+18) :: GUS (Takaiwa *et al.*, 1991a) were transferred to tobacco plants mediated by *Agrobacterium* infection (Fig. III-1). The transformants were selected by Kanamycin, (100 mg/L), and confirmed by PCR method. Subsequently, a series of the 5' deleted *GluA-3* :: LUC construct described as Chapter II (d897, d839, d760, d660 and d437; Fig. II-1) were introduced into the leaf disc of the progeny (R₁') of the *GluA-2* :: GUS or *GluB-2* :: GUS transformant. The double transformed tobacco plants were selected by bialaphos (10 µM) and Kanamycin (100 mg/L), and confirmed again by PCR method. These were transferred into nutrient soil and grown in a fully environmental controlled greenhouse at 14-h light (500 µE/cm², 25°C) and 10-h dark (23°C) cycle.

LUC and GUS assays and data analysis

Pods in various maturing stages, from each of 3 to 5 independent double transformed tobacco plants (R_0) and/or the progenies (R_1), was harvested at once or twice in a maturation period between 6 to 23 days after pollination (DAP). Those pods were frozen instantly in liquid nitrogen and stored at -80°C until use. Seeds from a quarter of the pod were collected and homogenized in liquid nitrogen, and mixed with 300 μl of LUC or GUS extraction buffer. The homogenate was centrifuged and the supernatant used for LUC or GUS assay, and total soluble protein assay (Chapter II).

LUC activity was rated as photon count per second per microgram of the total soluble protein. GUS activity was also normalized by a microgram of the total soluble protein. Untransformed plants were used as a negative control. LUC and GUS activities for each of the R_0 and R_1 plant not different from negative control at 95 % confidence level was considered LUC and GUS-negative. Only the LUC and GUS-positive plants were averaged for each construct, except for the constructs having no LUC and GUS-positive plants, and statistically analyzed by t-test.

Results

The LUC activities were first detected at six days after pollination (DAP) in all the R_0 and R_1 transgenic tobacco seeds containing the full length of 5' flanking region of the *GluA-3* gene (d897). The LUC activity increased rapidly and reached a peak at about 18 DAP (Tab. III-1) and then decreased to about 20% of the

maximum level by 23 DAP (Fig. III-2). The other 5' deletion clones of the *GluA-3* gene (d839, d760, d660 and d437) showed similar results other than the peak height.

On the other hand, the GUS activity directed by the *GluB-2* (-1209/+18) promoter was also first detected at 6 DAP. Subsequently, it reached the peak at about 18 DAP (Tab. III-1, Fig. III-2), and then decreased. The GUS activity regulated by the *GluA-2* (-1329/+1) promoter reached the peak at about 18 DAP as above described (18DAP), in spite of beginning of the detection was later than above described (9 to 10 DAP).

Every R_0 and R_1 tobacco seeds used for the LUC and GUS activity detection were from the same pods. The results from the R_0 and R_1 tobacco seeds were resembled each other in terms of the temporal specificity other than in the quantitative regulation.

These results indicate that the developmental expression of the restricted *GluA-3* promoters (-897/+11~-437/+11) maintain their temporal specificity in tobacco, and was similar to that of the other glutelin promoters.

DISCUSSION

The temporal expression of storage protein gene promoters during seed development is usually quite similar in the mother plant and in transgenic tobacco (Benfey and Chua, 1989). Sengupta-Gopalan *et al.* (1985) indicated that the phaseolin in tobacco behaves through the accumulation to the utilization in a manner similar to that in bean in both structural and biological properties. Leisy *et al.* (1989) and Takaiwa *et al.* (1991b) previously observed

the temporal expression of the *GluA-3* and *GluA-2* in transgenic tobacco plants, and suggested that the gene expressions were similar in rice and tobacco plants. These observations indicate the conservation of DNA regions specifying tissue- and temporal specific expression across evolutionarily diverse botanical families. The author confirmed that changes in the LUC activity in all the R₀ and R₁ tobacco seeds, controlled by the full length of the *GluA-3* 5' flanking region (d897), followed above observations.

On the other hand, the *GluA-3* mRNA reached its maximum level of expression earlier than the other glutelin genes (Okita *et al.*, 1989; Takaiwa and Oono, 1991c; Kim *et al.*, 1993). Zhao *et al.* (1994) showed that removal of 219 bp from -955 (-945) to -732 (-726) of *GluA-3* resulted in a reduction of overall promoter activity and a shift in temporal expression from a maximum of 16-20 days after flowering to 24 days (Numbers in parenthesis are base-pairs relative to the transcriptional start site of Gt3 as indicated by Zhao *et al.*, 1994). However, the temporal expression pattern of the LUC activities, those controlled by any of the deleted *GluA-3* 5' flanking region (d839, d760, d660 and d437), could not be changed by the deletion. Moreover, the internal control of the GUS activity directed by the *GluA-2* or *GluB-2* promoter showed similar temporal expression pattern to the LUC activity regulated by those *GluA-3* promoters. These results suggested that the temporal specific regulation of the *GluA-3* promoter may not reside within the sequence at least between -897 and -437. Taken together, one reasonable explanation is that the sequence required for the early stage specific expression of *GluA-3* is between -955 and -897.

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Summary

The double transformation analysis, in which the two different constructs of *GluA-3*/LUC and *GluA-2*/GUS or *GluB-2*/GUS are transferred into single explant, were used to demonstrate the difference between the temporal regulation of two promoters, *GluA-3* and *GluA-2* or *GluB-2*. The seed developmental stage specific expression of *GluA-3* was similar in rice and in transgenic tobacco. The 5' deletion mutation between the position -897 and -437 did not affect the specificity. In addition, the temporal expression of the deleted *GluA-3* promoters was not different from that of the internal control consisting of *GluA-2* and *GluB-2*. It was indicated that the temporal specificity may be regulated by an element in the region downstream from position -897. If the element(s) responsible for the temporal specificity were in the sequence between -897 and -437, the regulation would not be done by the single task of the sequence.

CHAPTER IV

Specification of the motifs in the proximal region responsible for endosperm-specific expression by the site-specific mutagenesis

In general, the *cis*-acting elements responsible for the basal gene expression are existing within the region between position -250 and -30 from the transcription start site (Tamura, 1995). In the rice glutelin gene family, the proximal region of the promoter may also play an important role in their expression. Kim and Wu (1990) demonstrate that at least six protein-DNA complexes are formed between the *GluA-2* promoter and nuclear protein factors, and five of the six are in the proximal region. It is shown that the proximal region of each of the different glutelin subfamily gene, *GluA-2* (Zheng *et al.*, 1993) and *GluB-1* (Takaiwa *et al.*, 1996), confer the endosperm specific expression by the 3' and the internal deletion or the substitution mutation experiments, respectively. It is noteworthy that two conserved sequences among the glutelin gene family, the AACA motif and the GCN4 motif, are identified in this region. Furthermore, as shown in Chapter II, the LUC activity in each of three 5' deletion constructs of the *GluA-3* promoter, d317 (-317/+11), d221 (-221/+11) and d138 (-138/+11), was extremely low, but still above the background. Of course, the conserved motifs in the glutelin gene, the AACA motif (-80/-61) and the GCN4 motif (-103/-93), are included in the regions listed above. In spite of those observations, the previous reports using any of the glutelin genes could not show the direct evidence for the role of those two conserved sequences.

In this Chapter, the author clearly showed by gain-of-function experiments that the 45-bp proximal region of the *GluA-3* promoter (-104/-60), including the AACCA motif and GCN4 motifs, is sufficient to confer the endosperm-specific expression.

Materials and Methods

Construction, Plant transformation and cultivation

A 45-bp oligonucleotide and three types of mutagenized oligonucleotides of the *GluA-3* promoter (-104/-60) were synthesized with *Sau3A* I recognition site at both the 5' and 3' end. Two putative *cis*-elements, the AACCA and GCN4 motif, were substituted to create *Apa* I and *Sma* I recognition sites, respectively. These were blunted their *Sau3A* I sites and cloned into the *Hinc* II site of the plasmid pUC18. After digestion with *Hind* III and *Bam*H I, fragments including the 45-bp oligonucleotides were excised from the plasmid and then subcloned into the upstream region of the CaMV 35S truncated promoter (-90/+9) in pLP19 binary vector (Szabados *et al.*, 1990) as shown in Figure IV-1. Orientation and accurateness of the inserted oligonucleotides were determined by sequencing (Sanger *et al.*, 1977).

The plasmid pLP19 containing these synthesized oligonucleotides were introduced into tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) by the *Agrobacterium* infection. The infected tobacco leaf disks were cultured and regenerated on the nutrient medium including Kanamycin (100 mg/L). Subsequently, the transformants were confirmed by Southern hybridization (Maniatis

et al., 1982) and PCR method (data not shown). All transgenic plants used for the analysis carried intact chimeric genes with various copy numbers. After regeneration, all the transformants were transferred into nutrient soil and grown in a greenhouse. These transformation and regeneration procedure are particularly described in Chapter II.

GUS assays and data analysis

Pods, leaf disks, pieces of stem and root of the transformants were frozen until use. GUS assays were performed as described by Jefferson *et al.* (1987). Total protein content was determined to normalize the GUS activities by the method of Bradford (1976). Data are shown for each transgenic line separately, and statistically confirmed the significance in the mean difference by t-test. For histochemical staining of GUS activity, hand-cut section of the seed was dipped into X-glucuronide solution for over night at 37°C. These procedure are also particularly described in Chapter II.

Results

It was first tested whether a 45-bp fragment of the *GluA-3* gene is sufficient to confer seed-specific expression to a truncated CaMV 35S promoter (-90/+9: -90Δ35S) /GUS construct. Subsequently, the functional importance of two putative *cis*-elements in the 45-bp fragment, GCN4 and AACA motifs, was examined by the substitutional mutations of individual elements as shown in Figure IV-2. The normal 45-bp construct (Nm) resulted in

2.6 folds induction of GUS activity as compared with that of the control level (-90 Δ 35S) in seeds, while it did not show any significant effects on the activities of non-seed tissues. It was notable that high GUS activity was observed in every tissue when the AACCA motif was mutagenized (MAM). The relative level of GUS activity to the control (-90 Δ 35S) in seeds, leaves, stems and roots were 4.3, 4.7, 1.9 and 2.3, respectively. Furthermore, when either the GCN4 (MGm) or both motifs (MAGm) was mutagenized, little activation was observed in every tissue.

To clarify the location of the expression sites directed by the 45-bp fragment, histochemical analysis was carried out as shown in Figure IV-3. The radicle of the embryo was stained in the -90 Δ 35S construct. Similar, although partially staining patterns in the radicle of the embryo were also observed in the constructs of MGm and MAGm. On the contrary, it was noteworthy that the endosperm tissue is specifically stained in the Nm construct, whereas whole tissue in the seeds are highly stained in the MAM construct.

In order to examine whether the promoter activity of the 45-bp fragment is dependent on the orientation, the normal and mutagenized 45-bp fragments (Nm, MAM, MGm and MAGm constructs) were changed in the reverse manner relative to -90 Δ 35S (Figure IV-1 and 4). The effect of the reverse orientation on the promoter activity was calculated as the relative GUS activity to that of corresponding forward construct. The reverse Nm and MAM constructs (rNm and rMAM) seemed to be decreased the relative GUS activity to about 0.7 and 0.5 in seed, respectively. On the contrary, the reverse MGm construct (rMGm) increased the relative GUS activity to about 4.0 in seeds. It was notable that the rMGm construct also remarkably enhanced the relative GUS activity in

leaves, stems and roots to about 12.1, 4.7 and 2.2, respectively. In non-seed tissues, the reverse orientation of Nm had little effect on promoter activity in any tissues other than leaf, in which it resulted in 2.3 fold increase. Furthermore, there was little difference in activity of non-seed tissues between the rMAM and MAM constructs. The rMAGm construct showed slightly lower activities than the MAGm construct in every tissues excepting root.

The author further examined if the GUS activity would be enhanced by the trimer of the 45-bp fragment. The trimer of the Nm fragment (Nt) showed almost the same effect as the monomer onto the -90 Δ 35S promoter activity. This tendency was similar in every tissue. The relative GUS activity of the trimer to the monomer fragment in seeds, leaves, stems and roots was about 0.9, 1.0, 0.9 and 1.0, respectively (Figure IV-5).

DISCUSSION

Both the AACA and GCN4 motifs are conserved in all the members of glutelin genes around -70 and around -100, respectively, suggesting that these elements should be considered as candidates for *cis*-regulatory element responsible for endosperm-specific expression. However, the previous 5'- deletion analysis of *GluA-3* has mainly indicated the functional importance of the upstream sequence between -346 and -263 (Zhao *et al.*, 1994) or -437 and -317 (Chapter II). In this chapter, it was tested whether a 45-bp fragment in the proximal region of the *GluA-3* gene (Nm: -104/-60) is sufficient to confer seed-specific expression when fused to a truncated CaMV 35S promoter (-90 Δ 35S). Subsequently, the

functional importance of two putative *cis*-elements in the 45-bp fragment, GCN4 and AACA motif, was examined by the substitutional mutation of each element. It can not be ruled out the possibility that the changes in expression the author observed here are not due to the oligonucleotides with which the author have substituted the original sequence, because these oligonucleotides are palindromic sequence and they could bind unknown factors. However, restriction site with palindromic sequence was commonly used to substitution mutation experiments in many published papers in order to assess the mutagenesis easily. Zheng *et al.* (1993) show the advantage of the substitution mutation using restriction site with palindromic sequence by double-gene vectors, in which a GUS reporter gene activity directed by a mutated test promoter is normalized over a LUC (luciferase) reporter gene activity directed by a wild-type reference promoter. Our results indicated that the small 45-bp fragment specifically enhances the activity of -90 Δ 35S in endosperm of transgenic tobacco. It is noteworthy that the mutation of the AACA motif (MAm) shows higher GUS activity than the Nm construct in every tissue, although the mutation of the GCN4 motif (MGm) and both motifs (MAGm) did not give any significant effect on the GUS activity. These indicated that the GCN4 motif generally enhances the promoter activity but the combination of the two motifs confers an endosperm-specific gene expression.

The GCN4 motif consists of the consensus seven nucleotide (TGAPuTCA) forming a palindromic structure, which is first recognized as the binding site for the yeast transcription factor GCN4 (Hill *et al.*, 1986) and the mammalian transcription factor jun and AP1 (Curran and Franza, 1988). It is also recognized by a bZIP type *trans*-acting factor of plants (de-Pater *et al.*, 1994; Hammond-

Kosack *et al.*, 1993). It has been reported that the GCN4 motif is implicated in some plant gene promoter activation in combination with other elements (i.e. endosperm motif : Müller *et al.*, 1993, 1995). de Pater *et al.* (1993) demonstrated that a trimer of a 22-bp fragment in the proximal region of the pea lectin promoter containing the GCN4 motifs confers seed-specific gene expression and GUS staining in whole seed tissues. However, it was shown here that the GCN4 motif enhances the promoter activity in every tissue or the enhancement is due to mutation of a putative negative regulatory element AACA (the MAm construct). This discrepancy may be explained by the difference in the CaMV 35S promoter used: de pater *et al.* (1994) used the -46 to +8 region (-46 Δ 35S) of the CaMV 35S promoter to express the oligomerized GCN4 motif, whereas the -90 to +9 region (-90 Δ 35S) was used in this study. The region between -90 and -46 of the 35S promoter includes a G-box which recognizes the b-ZIP *trans*-acting factor, TGA1a (Prat *et al.*, 1989; Katagiri *et al.*, 1989). Therefore, it was suggested that the combinatorial interaction between the G-box and GCN4 motifs may directed the expression in every tissue (Benfey *et al.*, 1989; Benfey and Chua, 1990). It is interesting to note that our 45-bp fragment, containing the GCN4 and AACA motifs, could not enhance the activity of the core -46 Δ 35S promoter (data not shown). Consequently, it is suggested that the synergistic interaction of GCN4, AACA and G-box may determine the distinctive seed specificity while the interaction between GCN4 and G-box may enhance the promoter activity in every tissue. The CATGCAT sequence, named RY element, is necessary but sufficient for the embryo-specific expression of leguminous seed storage protein genes (Bäumlein *et al.*, 1992; Lelievre *et al.*, 1992), such specificity

is also determined by the RY element in conjunction with other less defined elements (Fujiwara *et al.*, 1994). Salinas *et al.* (1992) showed that the tetramer of G-box sequences confer seed-specific expression when fused to -90 Δ 35S. Kawagoe *et al.* (1994) reported that the synergism between the G-box and CACCTG elements is required for activation of embryo-specific expression of the bean β -*phaseolin* promoter.

It is shown here that the endosperm-specificity is clearly determined by a set of the GCN4 and AACA motifs when fused to -90 Δ 35S. However, little is known about the function of the AACA motif. More than one AACA motif are repeated in the 5' flanking region of some glutelin genes, in addition to the AACA motif around -70 conserved in all the glutelin genes (Takaiwa *et al.*, 1991, 1996). Homology search of the AACA motif indicates that the sequence of the AACA motif is very similar to that of a gibberellic acid response element (Huang *et al.*, 1990; Skriver *et al.*, 1991; Gubler *et al.*, 1992; Rogers and Rogers, 1992). It was demonstrated by the gain-of-function experiment that an AACA motif located in the upstream region of *GluA-3* acts as a specific negative regulator suppressing the expression in tissues other than endosperm (Chapter II). Furthermore, it was interesting to note that the negative effect of the AACA motif is converted to a positive one when the 45-bp fragment is fused to -90 Δ 35S in reverse orientation with respect to the TATA box (rMGm), while the positive effect of the GCN4 motif is little affected besides seed by changing the orientation (rMAm). These results suggest that a set of the AACA and GCN4 motif does not function as one large orientation-dependent enhancer. It is also suggested that the effect of the AACA motif depends on the orientation of this motif and/or its position against the other

elements. However, it could not simply explain that the same reversed AACCA motif enhances the promoter activity only in rMGm construct but not in rNm construct. It is possible that the presence of the GCN4 motif in the 45-bp fragment affects the intensity of the interaction between the AACCA motif and the other.

In conclusion, it was clearly demonstrated that one set of the AACCA and GCN4 motif plays an important role in the endosperm-specific gene expression of the *GluA-3* gene, while the set alone did not result in high level gene expression. Consequently, the level of gene expression may depend on the combination with other sequences, such as endosperm motif and G-box motif in the upstream region. It is interesting to note that a combination of one set of the AACCA and GCN4 motifs and an endosperm motif or G-box motif is found in the 5'-flanking region of *GluA-3*, whereas two sets of the AACCA and GCN4 motifs are found within the 250 bp of 5'-flanking region of the *GluB* subfamily genes (Takaiwa et al., 1991, 1996). The author have recently shown by the 5'-deletion analysis of *GluB-1* gene that the two set of the AACCA and GCN4 motifs are required to confer the endosperm-specific expression (Takaiwa et al., 1996). It is possible that the difference in motifs combined with one set of the AACCA and GCN4 motif makes a difference in the gene expression level between the two glutelin gene families.

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Summary

A 45-bp proximal region of the rice glutelin promoter (-104/-60) containing two putative *cis*-elements, the AACCA motif and GCN4 motifs, was fused to a truncated CaMV 35S promoter (-90/+9; -90 Δ 35S) /GUS. The 45-bp fragment specifically enhanced the promoter activity in endosperm tissue of transformed tobacco. A substitution mutation of the GCN4 motif reduced the promoter activity, whereas the mutation of the AACCA motif increased the activity in the embryo as well as the endosperm. These results

suggest that the GCN4 motif generally enhances the promoter activity but the combination of the two motifs confers the endosperm specificity. Furthermore, the function of the two motifs was dependent on the orientation and/or distance from a G-box element in -90Δ35S, suggesting that the synergistic interaction between the factors that recognize those motifs and the G-box element is important for the transcriptional regulation.

CHAPTER V

Assessment of common regulatory regions required for the endosperm-specific expression of the glutelin genes

The observations in Chapter II and IV support the hypothesis that consensus sequences of glutelin genes may be involved in the endosperm-specific expression of the glutelin genes. It was further suggested by substitution mutations of these motifs that combinatorial interactions of these elements may be related to the specific regulation of the glutelin gene expression. However, the promoter activity of the proximal region is remarkably low and additional coupling elements are required to confer high levels of gene expression. In view of the fact that *cis*-regulatory elements of many specific genes are arranged in modules of short DNA segments (Benfey and Chua, 1990; Shen and Ho, 1995), specific expression of seed storage protein genes may also depend on combinatorial arrangements of distinct regulatory modules. Recent observations suggest that seed-specific expression is controlled by the interactions between two or more *cis*-elements (Bustos *et al.*, 1991; Herschlag and Johnson, 1993; Müller and Knudsen, 1993; Kawagoe *et al.*, 1994; Nunberg *et al.*, 1994). Therefore, it is necessary to examine the interactions between the motifs to understand the regulatory mechanism(s) of glutelin gene expression.

In this chapter, hybrid promoters were constructed, using the distal and proximal sequences of two glutelin genes (*GluA-3* and *GluB-1*) belonging to different subfamilies, to identify the common

regulatory mechanism(s) between them. These were transcriptionally fused to the LUC or GUS reporter gene and introduced into tobacco. A similar approach using hybrid promoter has been utilized in the identification of *cis*-regulatory regions involved in differential expression of *Arabidopsis* 2S albumin genes in the cotyledon (Conceição and Krebbers, 1994). Results showed that the proximal region was exchangeable between the two glutelin genes, suggesting that the endosperm-specific expression found in the two glutelin genes was primarily controlled by the proximal region containing the AACAA and GCN4 or G-box. Combinatorial interactions of *cis*-regulatory elements were also observed.

Materials and Methods

Construction, Plant transformation and cultivation

Hybrid promoters were constructed, which were composed of the 5' nested deletions of the *GluA-3* gene (PA1, -138/+11; PA2, -221/+11; PA3, -317/+11) or the *GluB-1* gene (PB1, -113/+18; PB2, -197/+18) as a proximal promoter and the PCR amplified fragments of the *GluA-3* gene (DA1, -438/-316; DA2, -438/-378; DA3, -385/-316) or 3' nested deletions of the *GluB-1* gene (DB1, -573/-145; DB2, -573/-178; DB3, -573/-227; DB4, -573/-265; DB5, -573/-342; DB6, -573/-424) as a distal region. These 5' flanking regions of the *GluA-3* gene (pREX21) (Takaiwa *et al.*, 1991a) and the *GluB-1* gene (pKpn9) (Takaiwa and Oono, 1991c) were subcloned from genomic clones of rice (λ INE21 and λ INE9, *Oryza sativa* L. cv. Mangetsu-mochi) and deleted or amplified as described in Chapter

II and Takaiwa *et al.* (1996). Thus, nine *GluA-3* distal and *GluA-3* proximal (Fig. V-1), six *GluA-3* distal and *GluB-1* proximal (Fig. V-3) and twelve *GluB-1* distal and *GluA-3* proximal (Fig. V-5) hybrid promoters were constructed.

These hybrid promoters were cloned into *Hind* III and *Bam*H I sites in the binary vector pGPTVL-bar or pBI101 that was followed by a firefly luciferase (LUC) or β -glucuronidase (GUS) reporter gene as shown in Fig. V-1 and Fig. V-4. The pGPTVL-bar is reconstructed from the pGPTV-bar binary vector (Becker, *et al.*, 1992) by conversion of the GUS to LUC as the reporter gene at the same restriction enzyme sites.

The plasmid pGPTVL-bar and pBI101 containing these synthesized oligonucleotides were introduced into tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) by the *Agrobacterium* infection. The infected tobacco leaf disks were cultured and regenerated on the nutrient medium including bialaphos (10 μ M) or Kanamycin (100 mg/L). Subsequently, the transformants were confirmed by Southern hybridization (Maniatis *et al.*, 1982) and PCR method (data not shown). After regeneration, all the transformants were transferred into nutrient soil and grown in a greenhouse. These transformation and regeneration procedure are particularly described in Chapter II.

LUC and GUS assays and data analysis

Pods and leaf disks of the transformants were frozen until use. GUS assays were performed as described by Jefferson *et al.* (1987). Total protein content was determined to normalize the GUS activities by the method of Bradford (1976). The distributions of

values for GUS and LUC activities were compared among plants carrying each construct to determine differences in expression between constructs. The two-sample *t*-test was used to compare the statistical significance between two constructs. These procedure are also particularly described in Chapter II.

Results

Effect of the hybrid promoters consisting of GluA-3 distal sequences and GluA-3 or GluB-1 proximal promoters

All three distal sequences of the *GluA-3* gene (DA1, -439/-316; DA2, -439/-378; DA3, -385/-316) enhanced the proximal promoter activities of the *GluA-3* gene (PA1, -138/+11; PA2, -211/+11; PA3, -317/+11) in the maturing seed of transgenic tobacco plants (Figs. V-1 and V-2). It was demonstrated that DA1 was most effective among the three distal sequences. Hybrid promoters using DA3 showed a small but significantly higher activity than the individual proximal promoters used for fusion. The enhancement due to DA1 seemed to be almost the same as the additive effect of DA2 and DA3 in every combination with PA1, PA2 and PA3 (Fig. V-2). It was notable that the activities of the hybrid promoters, consisted of DA1 and the individual three proximal promoters of the *GluA-3* gene, were also the same level as that of the *GluA-3* native promoter between positions -437 and +11. Furthermore, each of the three distal sequences of the *GluA-3* gene caused little change in their effects, irrespective of the combination with the proximal promoters, PA1, PA2 and PA3 (Fig. V-2). This was expected

because PA1, PA2 and PA3 are similar in their conserved motif sequences.

The enhancing effects of the three distal sequences of the *GluA-3* gene on the proximal promoter activity of the *GluB-1* gene between positions -113 and +18 (PB1) was very similar to those found in the hybrid promoter activities using three proximal promoters of the *GluA-3* gene ($p>0.10$) (Fig V-2, V-3 and V-4). On the other hand, much difference was shown in enhancing effect when the three distal sequences of the *GluA-3* gene were fused to the region between positions -197 and +18 of the *GluB-1* gene (PB2): the effect of DA1 was about three-fold higher than the sum of the individual effect of DA2 and DA3 (Fig. V-4). It should be noted that individual effect of DA1 and DA2 fused to PB2 showed about 11- and four- fold higher than those observed in the corresponding construct fused to PB1, respectively. However, there is little difference in enhancing effects in the case of DA3 between PB1 and PB2 used for the hybrid combinations. The activities of the hybrid promoters using DA3 seemed to be almost similar to those of the individual proximal promoters.

GluB-1 distal region includes multiple cis-acting regulatory elements governing the quantitative expression in seed

Distal regions of the *GluB-1* gene (DB1, -573/-145; DB2, -573/-178; DB3, -573/-227; DB4, -573/-265; DB5, -573/-342) enhanced the promoter activities of the *GluA-3* proximal region (PA1, -138/+11) (Figs. V-5 and V-6). The enhancing effect of DB1 was the highest among all constructs, and those of the others besides DB5 decreased according to the length of the 3' deleted

regions when fused to PA1. As shown in Figure V-6, the promoter activities directed by DB2, DB3 and DB4 were decreased to 67, 16 and 3 %, respectively, compared to the level of DB1. However, the promoter activities directed by DB5 was almost the same as that of DB4 (about 5 % of the level of DB1). The promoter activity by the shortest sequence (DB6) was the same as the control level (the activity of the PA1 alone). Both levels were less than 2 % of that of DB1.

On the other hand, although the *GluB-1* distal regions sufficiently activated the promoter activity of the region between positions -317 and +11 of the *GluA-3* gene (PA3), such effects were considerably different from that obtained by the combination with PA1 (Figs. V-5 and V-6). DB1 had the highest effect among all the deletion series, and the level of the enhancing effect was similar to that obtained by the combination with PA1. However, changes in the enhancing effect of the other *GluB-1* distal regions were not simply dependent upon the length of the 3' deleted regions as shown in results obtained by the combination with PA1. The promoter activities directed by DB2, DB3, DB4 and DB5 resulted in 6, 79, 5 and 21 % of that of DB1, respectively (Fig. V-6). It is interesting that the absolute values of the DB2 and DB5 effects combined with PA3 were much different from those combined with PA1. The DB2 effect combined with PA3 was about one-twentieth of that combined with PA1, while the DB5 effect combined with PA3 was about two-fold higher than that combined with PA1. The shortest *GluB-1* distal sequence (DB6) showed little effect to the promoter activity; it was about 3 % of the highest, and was comparable to the control level (the activity of the PA3 alone).

Tissue specificity

To investigate if any of the hybrid promoter constructs conferred an ectopic tissue specificity, the expression pattern of reporter gene directed by an individual hybrid promoter was also examined in the leaf of the transformants. In the hybrid promoters using *GluA-3* distal sequences, the levels of GUS expression in leaves were equal to those of negative (non-transformant) and positive (individual proximal sequences stand alone) controls for all the constructs (data not shown). On the other hand, in the hybrid promoters using *GluB-1* distal sequences, the levels of LUC expression in leaves were slightly higher than those of negative and positive controls for several constructs (Fig. V-7). LUC activity in leaves of PA3 constructs (DB1/PA3, 1.7; DB2/PA3, 1.3; DB3/PA3, 2.1; DB4/PA3, 1.0; DB5/PA3, 1.7; DB6/PA3, 1.3 cps/ μ g protein, respectively) seemed to be lower than those containing PA1 (DB1/PA1, 1.8; DB2/PA1, 2.4; DB3/PA1, 2.2; DB4/PA1, 2.6; DB5/PA1, 2.4; DB6/PA1, 1.6 counts per second (cps) per μ g protein, respectively). It was notable that the maximum of the averaged LUC activity in leaves was obtained when the DB3 (DB3/PA3, 2.1 cps/ μ g protein) or DB4 sequences (DB4/PA1 2.6 cps/ μ g protein) were used as a distal region of the hybrid promoter. Furthermore, significant differences between the levels of LUC expression in seeds and those in leaves were observed only in constructs using the following distal/proximal combinations of the sequence: DB1/PA1 ($p < 0.10$), DB1/PA3 ($p < 0.10$), DB2/PA1 ($p < 0.05$), DB3/PA1 ($p < 0.05$), DB3/PA3 ($p < 0.10$), DB4/PA3 ($p < 0.05$) and DB5/PA3 ($p < 0.01$).

DISCUSSION

All the distal regions of the *GluA-3* gene (DA1, DA2 and DA3) were effective in enhancing the promoter activities of the proximal regions of the *GluA-3* (PA1, PA2 and PA3; Fig. V-2) and *GluB-1* genes (PB1 and PB2; Fig. V-4). DA1 was most effective, and the effect seemed to be almost the same as the sum of the individual DA2 and DA3 effects in every combination with the *GluA-3* and *GluB-1* proximal promoters except for PB2. In other words, individual DA2 and DA3 alone do not seem to be sufficient to confer the full level of the DA1 effect when fused to the proximal region of glutelin genes. However, these results are incompatible with the observation in Chapter II, where DA1 and DA3 are almost equally effective and DA2 showed no effect or even a negative effect in activating the CaMV 35S truncated promoter (-90/+9; -90 Δ 35S) in the transgenic tobacco seeds. The discrepancy may be accounted for by differences in combinatorial interactions of *cis*-regulatory elements responsible for the seed-specific expression. Two G-box-like sequences (as-1; TGACG, -82/-78 and -70/-66) are considered as candidates for the interactive *cis*-regulatory elements in -90 Δ 35S, which is recognized by the b-ZIP type *trans*-acting factor (ASF-1) (Benfey *et al.* 1990; Katagiri *et al.*, 1989). On the other hand, the candidates of *cis*-regulatory elements in the *GluA-3* and the *GluB-1* genes, GCN4 motif (GTGAGTCAC, -102/-94 of the *GluA-3* gene and ACAAGCCAT, -99/-91 of the *GluB-1* gene) and AACA motif (AACAAACTC/ATATC, -77/-65 of the *GluA-3* gene and -73/-61 of the *GluB-1* gene), are observed in the 5' flanking region of both genes. The GCN4 motif in the proximal region of the *GluB-1* gene is critically distorted. However, this putative junk GCN4 motif might be replaced by a G-box-like motif (GTACGTGC) between positions -81

and -74, which may act as an important *cis*-element (Takaiwa *et al.*, 1996; Chapter IV). It has been reported that the GCN4 motif can be specifically recognized by b-ZIP type (G/C-hybrid type) Opaque-2 and TGA *trans*-acting factors (Holdsworth *et al.*, 1995; de Pater *et al.*, 1994). Furthermore, the AACCA motif (AACAAACTGCATT, -433/-421), a G-box-like motif (TGACGTGG, -386/-379), the GCAA motif (CCAAAATGA, -352/-344) and an endosperm-like motif (TTTAAAGT, -325/-318) are considered as candidates for the putative *cis*-elements in DA1 (Chapter II).

The interaction between the putative *cis*-elements in DA1 and those in the *GluA-3* proximal region might be independent of the distance between the elements, since three combinations using DA1 caused little change in the expression level when the proximal region was replaced by others (PA1, PA2 and PA3) of the *GluA-3* gene (Fig. V-2). The replacement of the proximal region giving little change in the hybrid promoter activity was also shown by the combinations using the other distal sequences of the *GluA-3* gene (DA2 and DA3), individually. These results indicate that the sequence between positions -317 and -138 of the *GluA-3* gene is not always implicated in the quantitative expression of the *GluA-3* gene. In addition, the replacement of the proximal region from PA1 to PB1 resulted in little effect on the hybrid promoter activities irrespective of the combinations with the three *GluA-3* distal sequences (Figs. V-2 and V-4). These results suggest that an essential regulatory mechanism responsible for the endosperm-specific expression may be in common with the *GluA-3* and *GluB-1* genes (Fig. V-8).

Sequence comparison between the *GluA-3* and *GluB-1* promoters revealed a limited number of conserved motifs in the

proximal regions. It has been recently indicated that a set of the conserved motifs in the proximal regions of the glutelin genes, the AACAA motif and GCN4 motif, is important for the endosperm specific expression (Takaiwa *et al.*, 1996; Zhao *et al.*, 1994, Chapter II). Consequently, it was suggested that the set of the conserved motifs in the proximal region may primarily determine the endosperm specific expression in both the glutelin genes. On the other hand, it has been demonstrated that the expression pattern of the *GluA-3* and the other glutelin genes is different from each other in the temporal manner of the mRNA (Okita *et al.*, 1989; Takaiwa *et al.*, 1991b). It has been also demonstrated that the temporal expression of the *GluA-3* gene may be involved in the sequence between positions -945 and -726, possibly in conjunction with the downstream sequences which mainly regulate the quantitative expression (Zhao *et al.*, 1994; Croissant-Sych and Okita, 1996). However, it is still unclear whether proximal elements of the *GluA-3* and *GluB-1* promoters relate temporal expression, and how the hybrid promoters affect it.

Furthermore, in hybrid promoters using PB2 as a proximal region, the effect of DA1 was three to four times higher than the sum of the individual DA2 and DA3 effect (Fig. V-4). This suggests that the sequence between positions -197 and -113 of the *GluB-1* gene might enhance the additive effect on at least two separated *cis*-elements in DA1 (Fig. V-8). The importance of *cis*-element(s) in this region responsible for the quantitative regulation has already been suggested by the 3' deletion analyses (Takaiwa *et al.*, 1996). The GCN4 motif (GCTGAGTCA; -165/-156 of *GluB-1*) is considered as a candidate for the *cis*-element. Such a higher effect than the additive effect of two or more *cis*-elements might be accounted for

by a more-than-additive effect due to synergistic interactions (herschlag and Johnson, 1993). Kawagoe *et al.* (1994) showed the typical synergism by three CANNTG motifs, CACGTG (G-box), CACCTG and CATATG, in the β -phaseolin gene. The combination between the two of the three motifs, the G-box/CACCTG, the G-box/CATATG and CACCTG/CATATG, induces three levels of transcription, namely, more-than-additive, additive, and less-than-additive, respectively. It might be possible that the GCN4 motif, the AACA motif and G-box-like motif in the glutelin hybrid promoters synergistically interact with each other.

On the other hand, five distal sequences of the *GluB-1* promoter (DB1, DB2, DB3, DB4 and DB5), which were progressively deleted from the 3'-end, also enhanced the proximal promoter activities of the *GluA-3* gene (PA1 and PA3) (Fig. V-6). DB1 showed the highest effect and the activities of the others besides DB5 decreased as the deletion proceeded, when fused to PA1. These results revealed that the *GluB-1* distal region between positions -424 and -145, includes multiple *cis*-elements involved in the quantitative regulation. Two AACA motifs (AACAAACCTACAA, -413/-401; AACAAACTCCATT, -212/-200), the GCAA motif (GCAAAAATGA, -352/-342) and GCN4 motif (GCTGAGTCA, -165/-156) are considered as candidates for the *cis*-elements. Similar results were also obtained from the 3' deletion analyses of *GluB-1* gene, in which the -90 Δ 35S was used as a proximal promoter (Takaiwa *et al.*, 1996). In addition, it was interesting that two of the five distal sequences (DB2 and DB5) showed quite different actions, when fused to PA3 (Fig. V-6). Effects of the two sequences fused to the PA3 sequence were about one-twentieth and twice of those obtained by the combination with PA1,

respectively, although the differences in the effect of the others combined with PA3 were not significant, compared to those obtained by the combination with PA1. The different effects of the *GluB-1* sequences obtained by the combination with PA3 might also be caused by change in the interactions between *cis*-elements in the distal and the proximal promoters. There are two possibilities for explaining the difference in the interaction: element(s) with reversible functions may exist between positions -227 and -178 and/or -424 and -342 of the *GluB-1* gene that can function in conjunction with either an enhancer or a silencer element, depending on the distance from the *cis*-elements between positions -138 and +11 of the *GluA-3* gene. Alternatively, a negative element may exist between positions -317 and -138 of the *GluA-3* gene which specifically interacts with a *cis*-element between positions -227 and -178 and positions -342 and -265 of the *GluB-1* gene. It has been observed that endosperm motif (Müller and Knudsen, 1993) and RY motif (Fujiwara and Beachy, 1994) change the function dependent of the interaction with the other element(s). A similar situation has been observed when a 45 bp of proximal region of the *GluA-3* gene, containing the AACAA and GCN4 motifs, was fused to the -90Δ35S in reverse orientation from the transcription start site (Chapter IV). These results also indicated that the set of the conserved motifs in the proximal region may essentially regulate the endosperm specific expression in both the glutelin genes.

In conclusion, the hybrid promoter analyses using two different subfamily glutelin genes, *GluA-3* and *GluB-1*, experimentally revealed the existence of common regulatory mechanism(s) for the endosperm-specific expression. A set of the conserved motifs in the proximal regions of both glutelin genes, the

AACA motif and GCN4 or G-box-like motif, is considered as candidate *cis*-elements primarily determining the endosperm-specific expression. Variations in the quantitative expressions between both the glutelin genes may be attributed to differences in the combination and/or the synergistic interaction between these motifs and the additional motifs in the distal regions. A similar observation has been done using sunflower helianthinin storage protein genes (Nunberg *et al.*, 1994). Thomas (1993) proposed the model of a bipartite organization of seed storage protein promoter, in which proximal promoter regions direct seed specific expression and more distal regions enhance and modulate the basic expression patterns conferred by proximal regions. Therefore, expression of many seed storage protein genes appear to be regulated by combinatorial interactions of multiple *cis*-regulatory modules. As a further study, site-directed mutagenesis of candidate motifs will be required to elucidate the combinatorial interactions or synergisms among *cis*-regulatory elements found in the rice glutelin genes in homologous background.

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Summary

Most of the rice (*Oryza sativa* L.) storage protein glutelin genes are coordinately expressed during seed maturation, although there is considerable divergence in the 5' flanking regions between members of *GluA* and *GluB* subfamilies. Hybrid promoters among the 5' distal and proximal sequences of *GluA-3* and *GluB-1* genes were constructed to elucidate the regulatory mechanism(s) responsible for their common endosperm-specific expression. The constructs were transcriptionally fused to the firefly luciferase (LUC) or β -glucuronidase (GUS) reporter gene and their activities were examined in maturing tobacco seeds and leaves. A set of

conserved motifs, the AACCA motif and GCN4 or G-box-like motif, in the proximal regions of both the glutelin genes was shown to be *cis*-elements required for endosperm-specific expression. In addition, it has been demonstrated that *GluA-3* and *GluB-1* differ in the interactions between the *cis*-elements in the distal and the proximal promoter regions, and this could lead to observed differences in the quantitative regulation between the glutelin genes.

CHAPTER VI

Concluding Remarks

First, the author clearly showed that the 5' flanking region of the *GluA-3* gene was consisted of two important regions responsible for the gene regulation: one was in the distal sequence between positions -897 and -437, and the other was in the proximal sequence between positions -104 and -60.

Second, the author described that the developmental expression pattern directed by the deleted *GluA-3* promoters (-897/+11~-437/+11) was similar to that of the other glutelin *GluA-2* and *GluB-2* promoters, when introduced into transgenic tobaccos containing the chimeric genes composed of the *GluA-2* or *GluB-2* promoter and the GUS reporter gene. These results suggested that the temporally different expression pattern of the *GluA-3* gene from other glutelin genes may not reside within the sequence at least between -897 and -437. If it were there, the regulation would not be done by the single task of the sequence.

Third, some of the consensus sequences of the glutelin genes were found out from each of the two regions of *GluA-3*. These individual region conferred the quantitative (-385/-316 and -102-94; GCN4 motif) and the spatial-specific expression (-439/-378 and -77/-63; AACA motif) to the CaMV 35S truncated promoter (-90 Δ 35S) in transgenic tobacco seeds.

Fourth, a putative role of these two important sequences in the *GluA-3* was basically similar to that observed in the CaMV 35S truncated promoter. In addition, the two separable distal sequences (-439/-378 and -385/-317) and the proximal sequence (-104/-60)

were additively interacted each other in the *GluA-3*.

Finally, an essential regulatory mechanism responsible for the endosperm-specific expression may be in common with the other glutelin genes. Variations in the quantitative levels among the glutelin genes may be attributed to differences in the combination and/or the synergistic interaction between these motifs and the additional motifs in the distal regions.

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Publications

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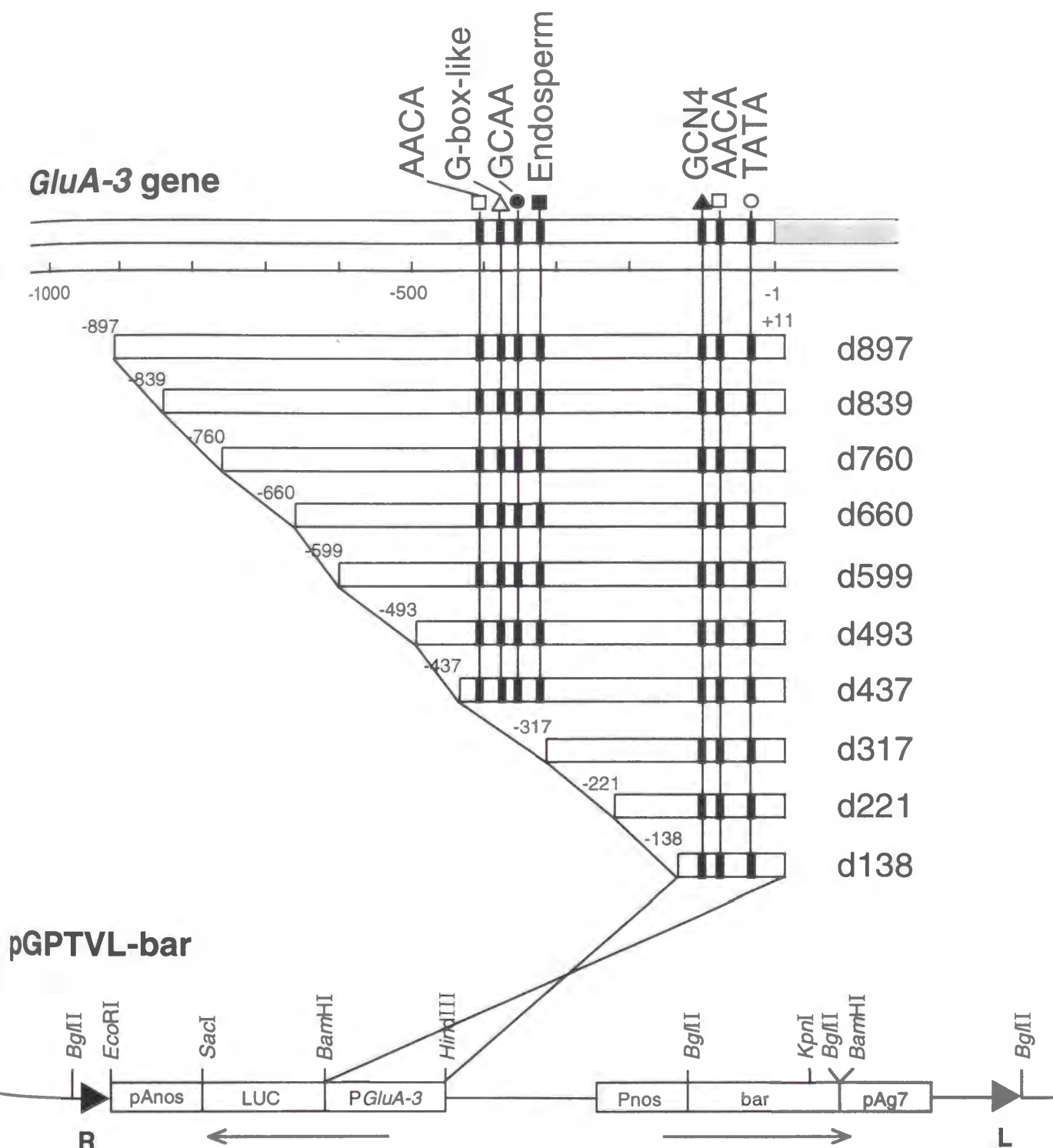


Figure II-1. Structure of the 5' deletion constructs and physical organization of the chimeric gene constructs used to transform tobacco plants. A series of 5' deletions of a 897 bp 5' flanking region of the *GluA-3* gene (-897/+11, relative to transcription initiation site) were generated. The motifs discussed in the text are marked by boldface vertical bars.

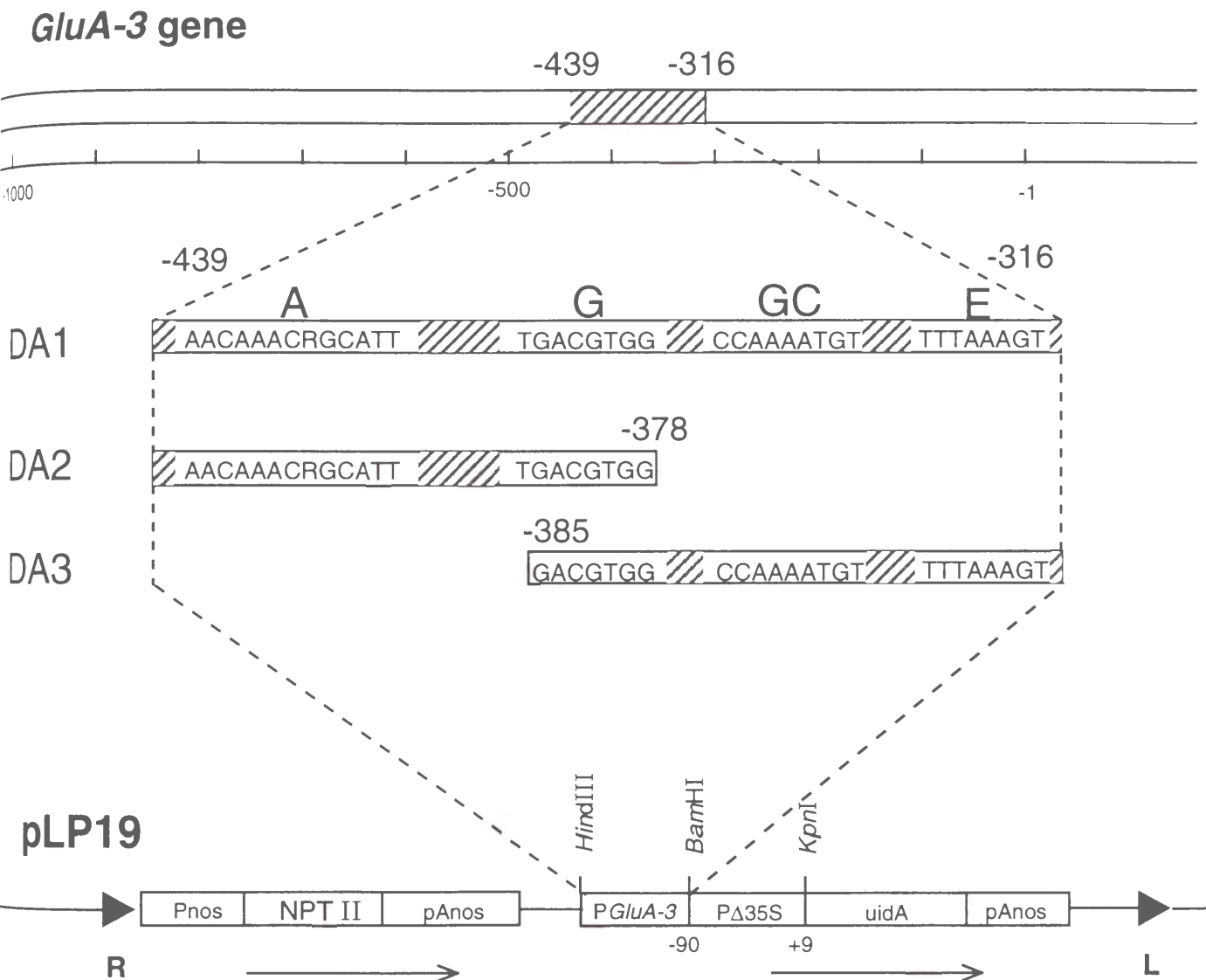


Figure II-2. Physical organization of the *GluA-3* putative enhancer regions and GUS reporter gene constructs used for the gain-of-function experiments. The motifs discussed in the text are indicated by the boldface characters; A, AACA motif (-433/-421); G, G-box-like motif (-386/-379); GC, GCAA motif (-352/-344); E, endosperm motif (-325/-318).

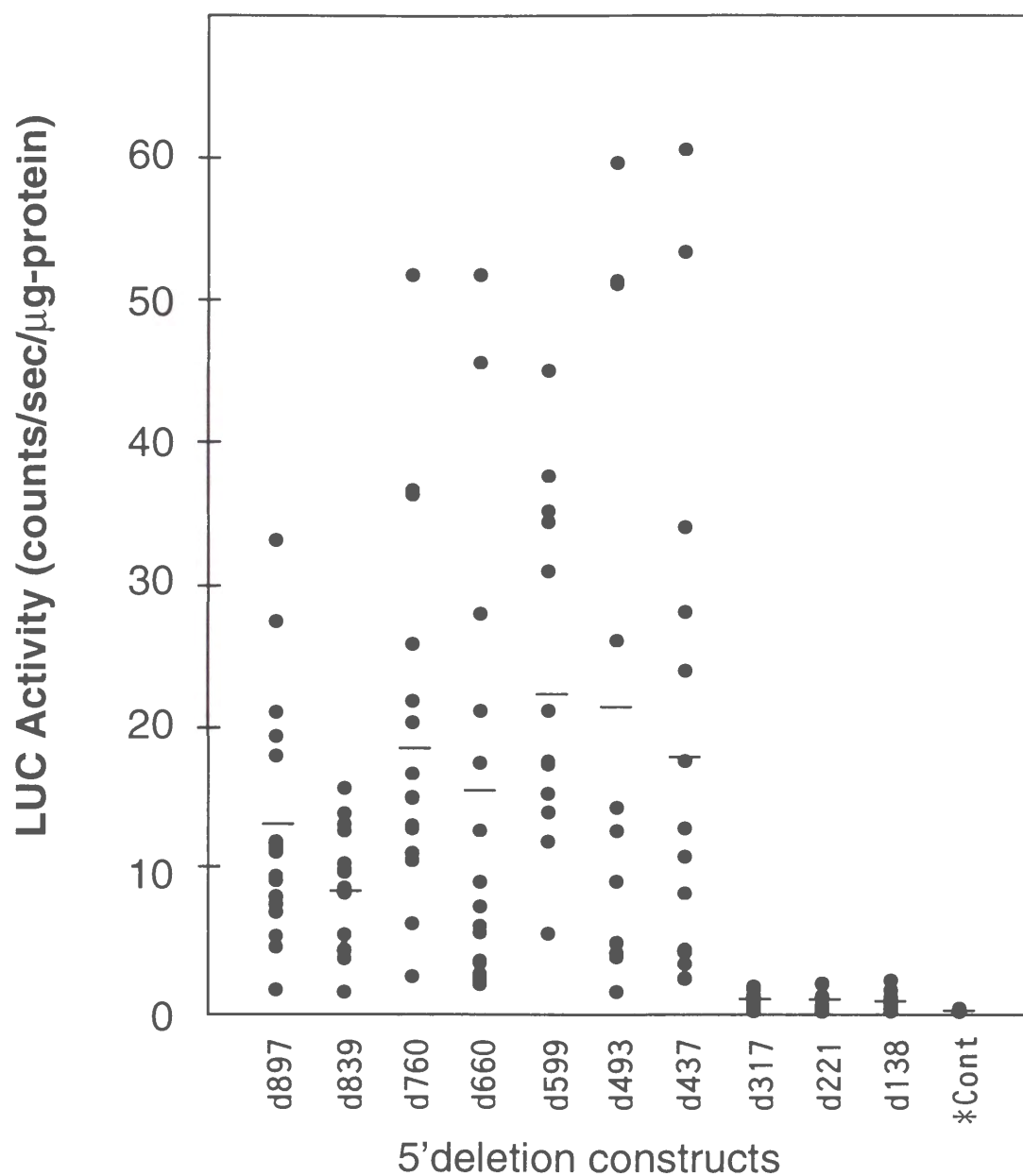


Figure II-3. Expression of the LUC gene in transgenic tobacco seeds containing *GluA-3* 5'-deleted-promoters::LUC. Constructs used in the study are shown in the Fig.II-1. The values indicated are average LUC activities with standard errors from 7-20 independent plants. Control means the values from non-transformants.

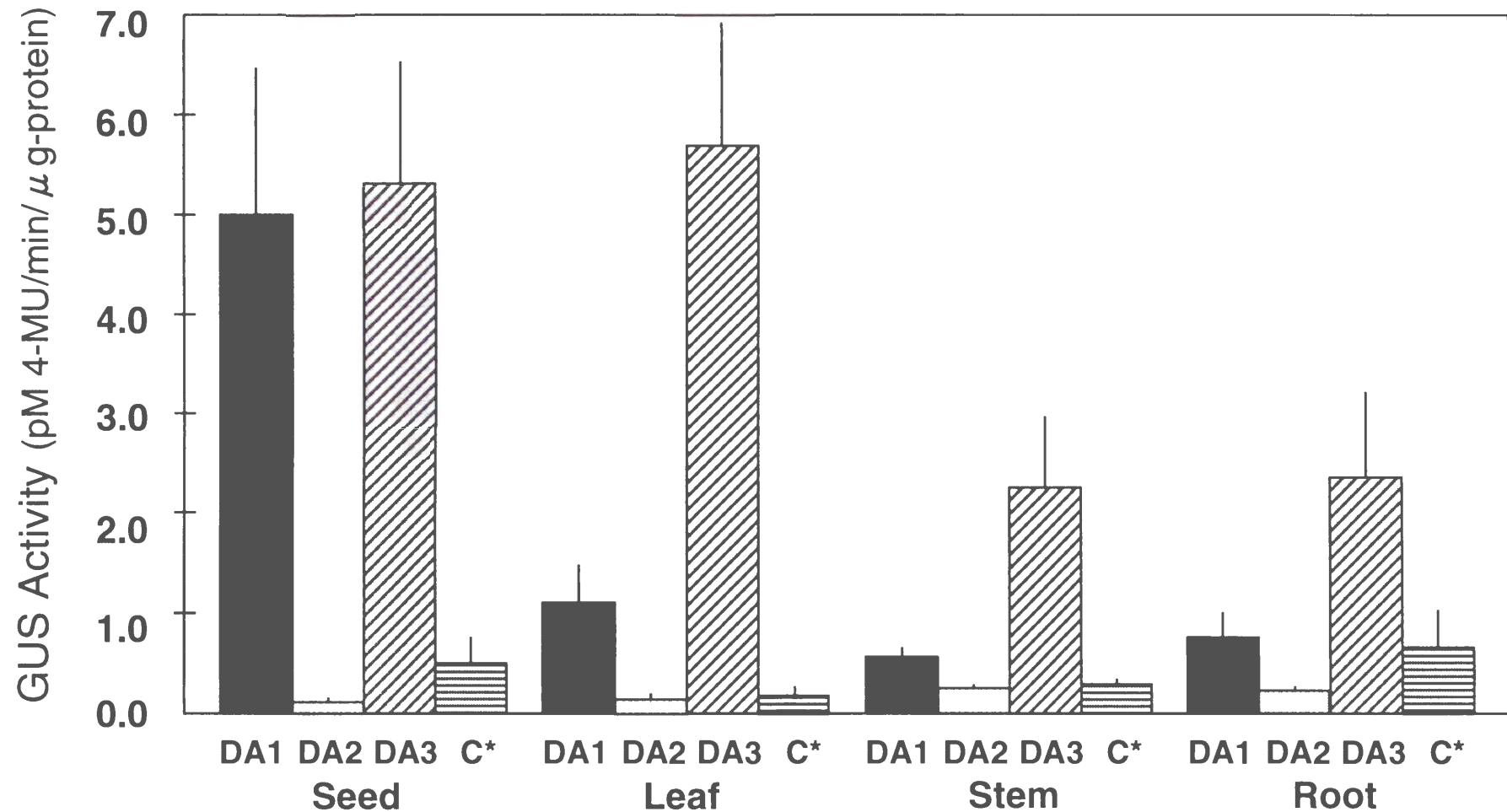


Figure II-4. The effect of the putative enhancer regions on the GUS activity of a truncated CaMV 35S promoter in seeds, leaves, stems and roots of transformed tobacco.

Constructs used in the study are shown in Fig. 2. The negative control (C*) refers to the activity of a truncated CaMV 35S promoter alone. Seeds were harvested between 16 and 18 days afterpollination for the assay. The values represented the mean from ten to 20 independent transformed tobacco plants per construct, and the standard error of each mean is indicated by the vertical bar.

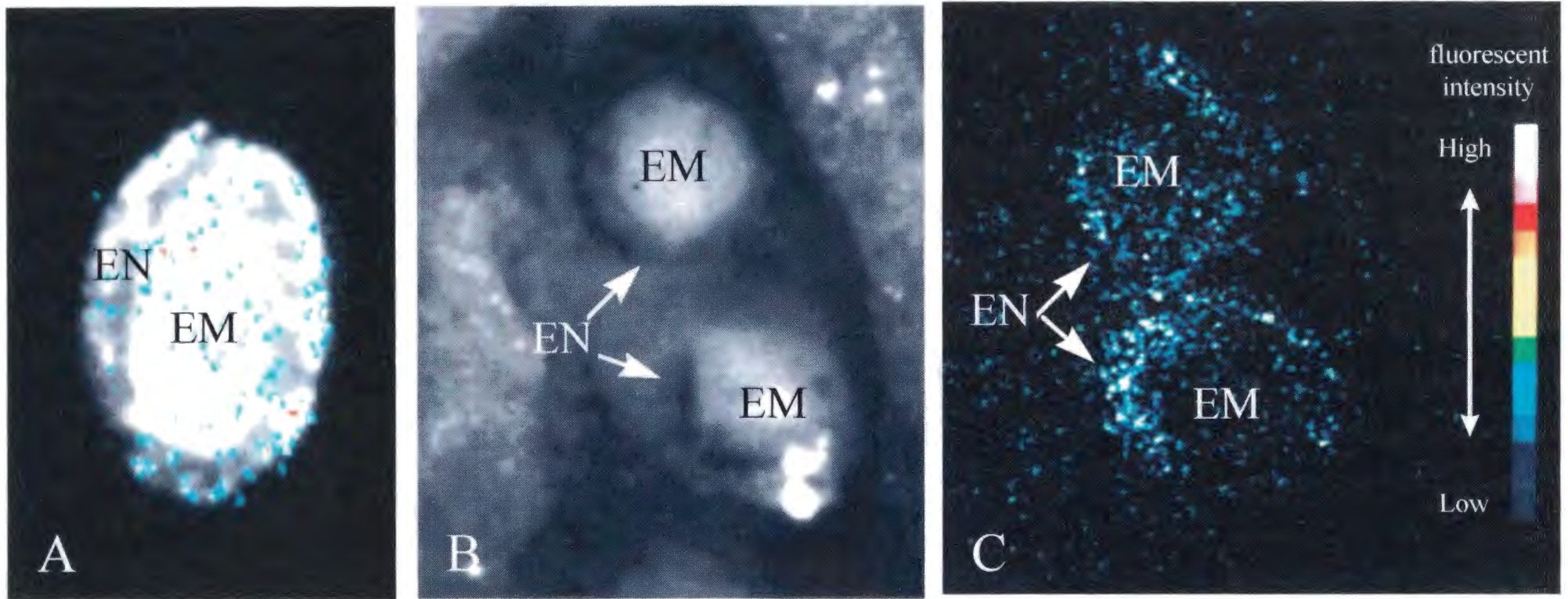


Figure II-5. Localization of LUC enzyme activity in endosperm tissue of the transgenic tobacco plants.

A, LUC luminescence of d138::LUC with a normal microscopic picture; B, a normal microscopic picture of d437::LUC; C, LUC luminescence of d437::LUC
EM, embryo; EN, endosperm.

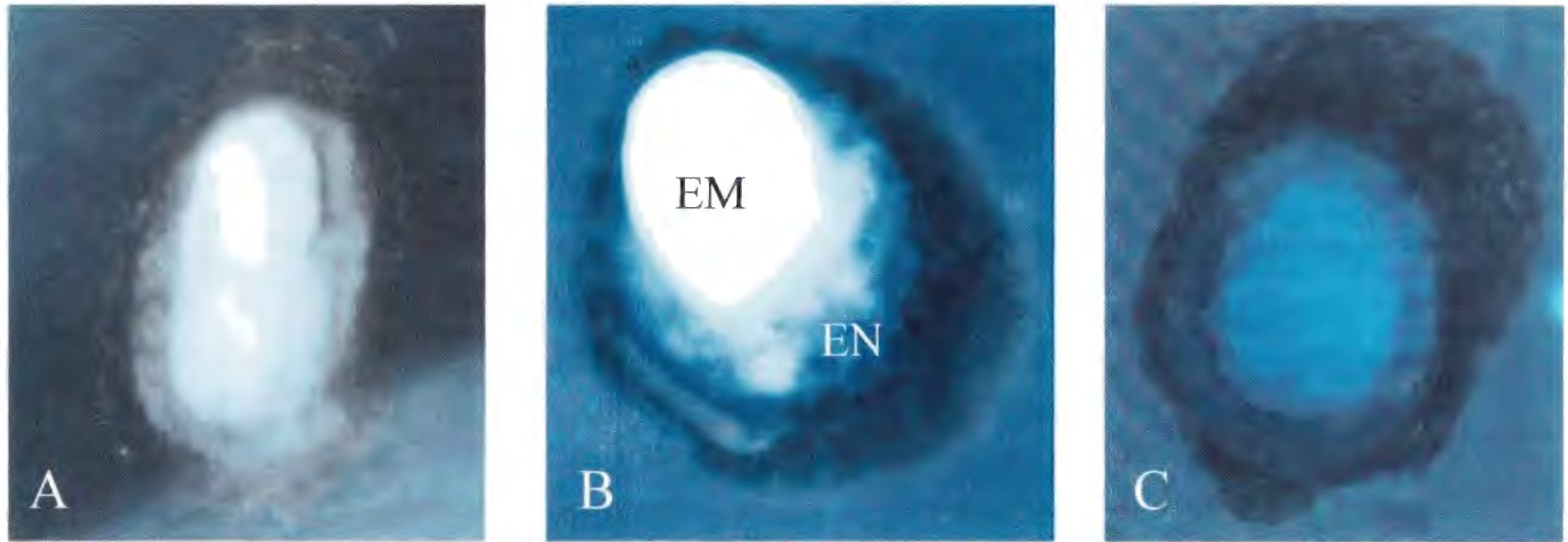


Figure II-6. Localization of GUS enzyme activity in endosperm tissue of the transgenic tobacco plants.

A, non-transformant; **B**, DA1::CaMV35S::GUS; **C**, DA1::CaMV35S::GUS
EM, embryo; **EN**, endosperm.

Table II-1. Expression of the LUC gene in transgenic tobacco plants containing *GluA-3* 5'-deleted-promoters::LUC.

Construct	No. of Plants	Seed	Leaf	Stem	Root
d897	18	12.7 ±1.9	1.1 ±0.1	1.0 ±0.4	1.3 ±0.3
d839	17	8.8 ±1.2	1.2 ±0.2	1.5 ±0.4	1.6 ±0.3
d760	18	18.7 ±3.3	1.6 ±0.3	1.8 ±0.5	1.6 ±0.4
d660	16	13.9 ±3.9	1.4 ±0.3	1.7 ±0.4	1.7 ±0.3
d599	13	22.4 ±3.5	1.7 ±0.4	1.5 ±0.4	1.7 ±0.4
d493	13	21.7 ±6.6	1.4 ±0.2	1.7 ±0.5	1.5 ±0.4
d437	15	18.1 ±4.8	1.2 ±0.1	1.6 ±0.4	1.6 ±0.3
d317	19	1.3 ±0.3	0.9 ±0.1	1.3 ±0.3	1.5 ±0.4
d221	17	1.2 ±0.3	0.9 ±0.1	1.1 ±0.2	1.3 ±0.3
d138	20	1.4 ±0.3	0.9 ±0.1	1.5 ±0.3	1.2 ±0.3
Control	7	0.3 ±0.0	0.5 ±0.2	1.3 ±0.4	1.0 ±0.2

Constructs used in the study are shown in the Figure II-1. LUC fluorescent intensity was measured as photon counting per second and expressed in per g protein. The values indicated are average LUC activities with standard errors from 7-20 independent plants. Control means the values from non-transformants.

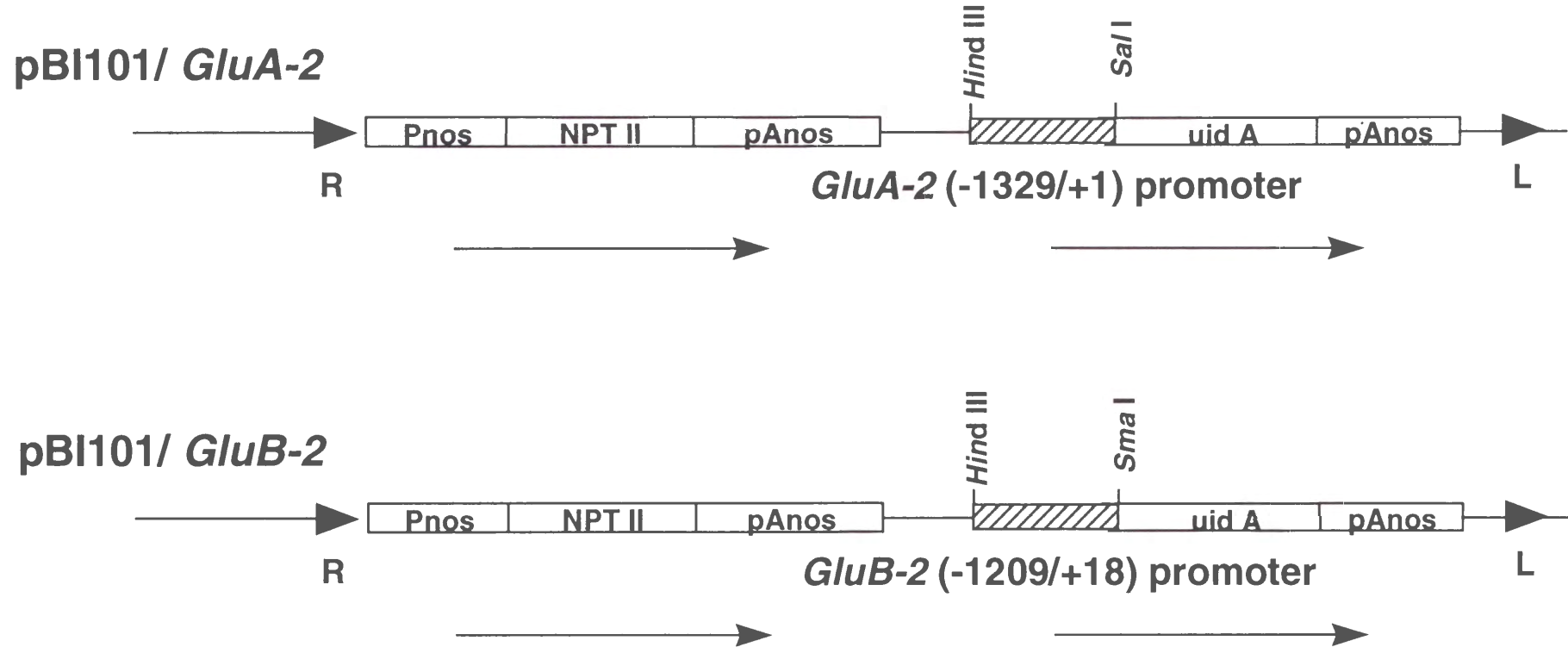


Figure III-1. Physical organization of the chimeric gene constructs used to the first transformation.

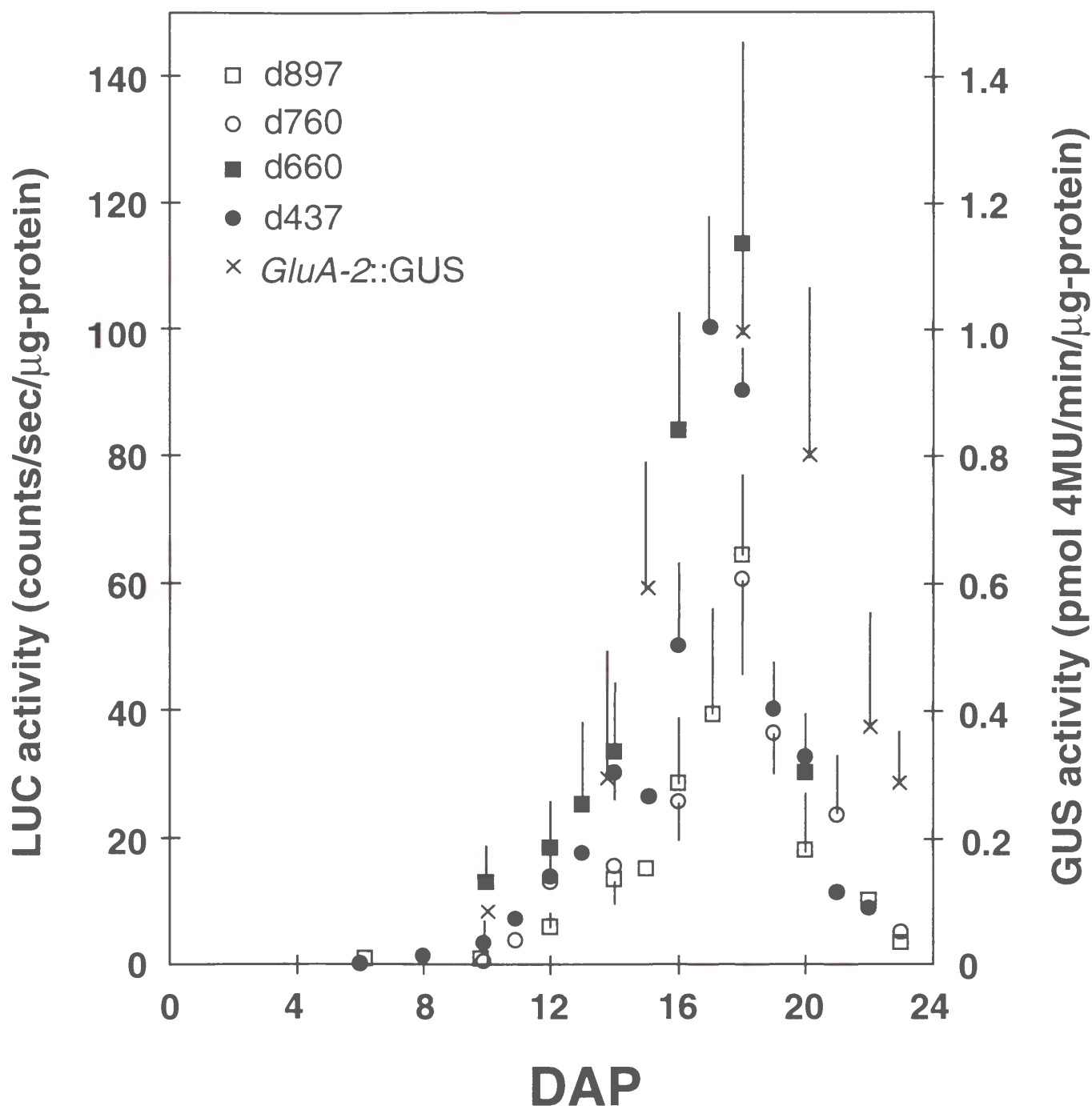


Figure III-2. Comparison of the temporal manner of *GluA-2* (-1329/+1)::GUS and *GluA-3* (-897~-437/+11)::LUC chimeric constructs in the developing seeds of *R₁* tobacco. The value of each point is the mean LUC or GUS activity obtained from one to five independent transformants for each construct. The vertical bar on the symbol indicates the standard error of each mean. Seeds from the same pods were used for the detection of each of the LUC and GUS activity regulated by d897 and the *GluA-2* promoter, respectively.

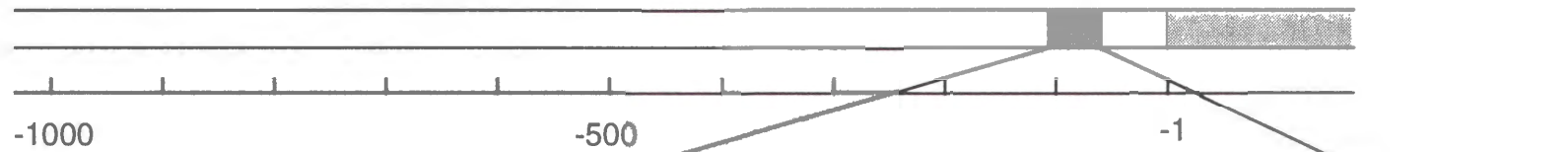
Table III-1. Temporal expression of the *GluA-3* (-897/+11)::LUC, *GluA-2* (-1329/+1)::GUS and *GluB-2* (-1209/+18)::GUS chimeric genes in the same R₀ and R₁ developing tobacco seeds.

Generation / Construct		DAP				
		6	10	14	18	22
R ₀	<i>GluA-3</i> ::LUC	1.9 ± 0.9	2.9 ± 1.8	11.2 ± 3.1	18.3 ± 5.2*	1.3 ± 0.3
	<i>GluA-2</i> ::GUS	-	0.05 ± 0.04	0.3 ± 0.1	0.6 ± 0.3*	0.3 ± 0.2
R ₁	<i>GluA-3</i> ::LUC	2.7	3.9	18.4 ± 2.3	64.3 ± 11.5*	12.9
	<i>GluA-2</i> ::GUS	-	0.08	0.3 ± 0.2	1.0 ± 0.3*	0.4 ± 0.1
R ₀	<i>GluA-3</i> ::LUC	2.1 ± 2.8	7.0 ± 4.8	10.2 ± 4.7	17.5 ± 7.4*	1.6 ± 0.3
	<i>GluB-2</i> ::GUS	1.0 ± 0.5	2.9 ± 0.8	11.8 ± 3.5	19.3 ± 7.5*	8.2 ± 3.3

Constructs used in the study are shown in the Figs. II-1 and III-1. The values indicated are average LUC and GUS activities with standard error from 1-5 independent plants. Every tobacco seeds used for the LUC and GUS activity detection were from the same pods. LUC activity was rated as photon counting per second per µg protein. GUS activity was calibrated against pmol 4-MU and normalized by µg protein. The maximum LUC and GUS activity within the maturation period was indicated by an asterisk.

A

GluA-3 gene



-104

-60

GCN4

AACA

TAGTCACTCACTTCATATGTGGACATTAACAAACTCTATCTTAAC

Normal (N)

TAGTCACTCACTTCATATGTGGACATTGGGCCCACTATCTTAAC

AACA mutant (MA)

TAGCCCGGGTCTTCATATGTGGACATTAACTCTATCTTAAC

GCN4 mutant (MG)

TAGCCCGGGTCTTCATATGTGGACATTGGGCCCACTATCTTAAC

**AACA/GCN4
mutant (MAG)**

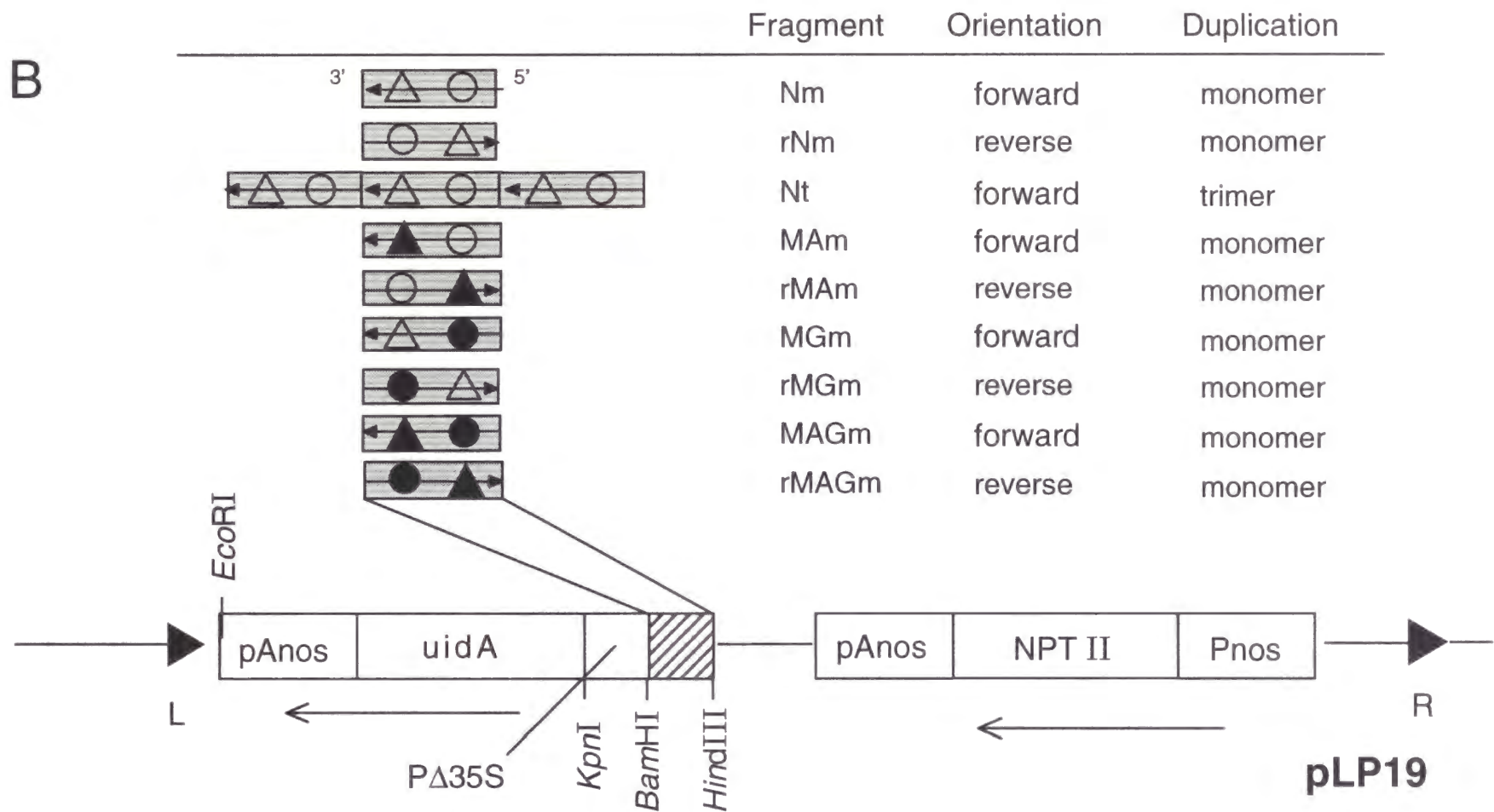


Figure IV-1. Construction of *GluA-3* :: -90 Δ 35S chimeric promoters.

A. Sequence of *GluA-3* synthetic oligonucleotides used for the chimeric promoters. B. Orientation and duplication of the *GluA-3* synthetic oligonucleotides and structure of pLP19 binary vector. Open circles and triangles indicate the normal GCN4 and AACA motifs, and closed circles and triangles indicate the mutated GCN4 and AACA motifs, respectively.

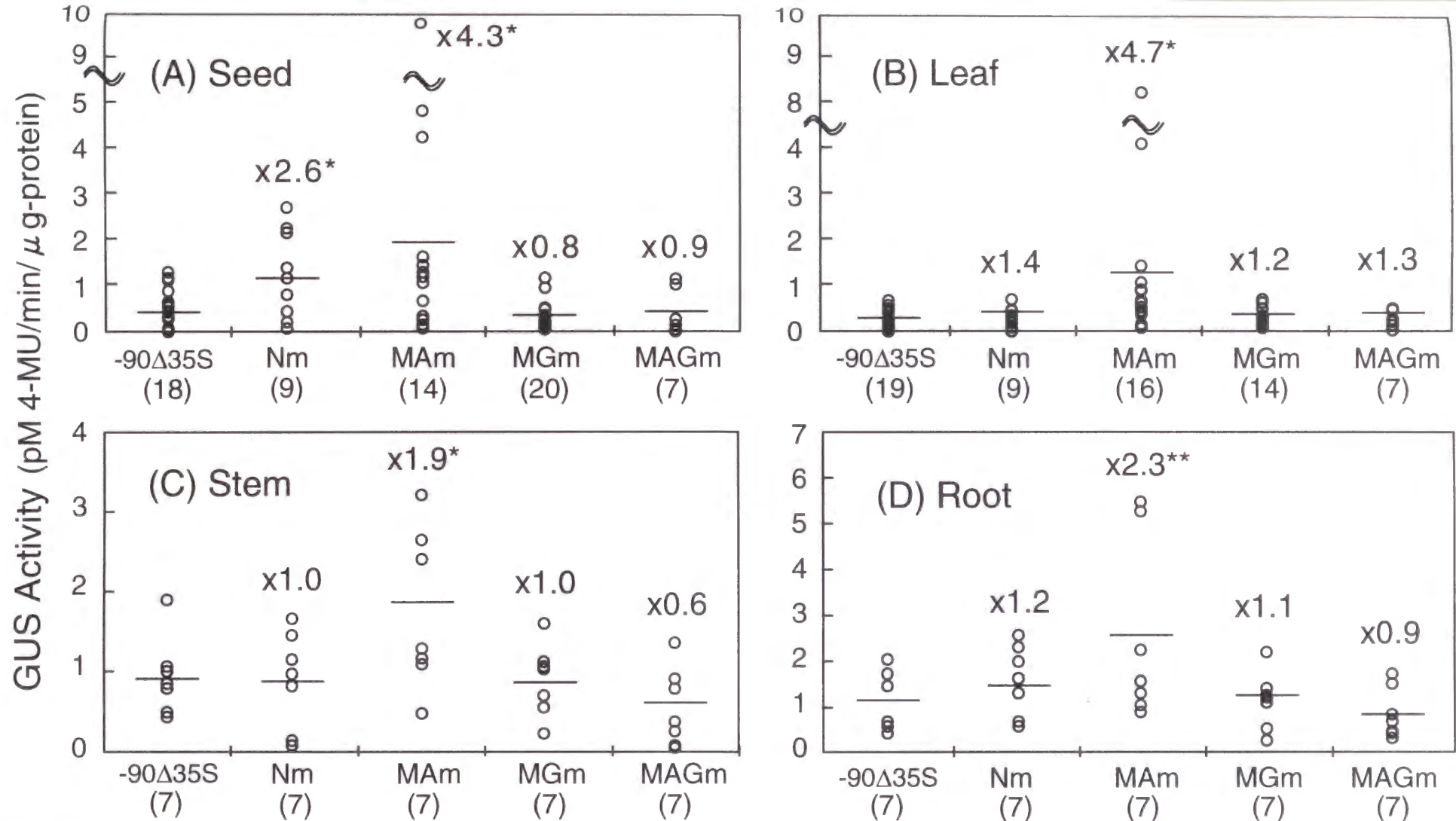


Figure IV-2. Functional analysis of the normal and mutated 45 bp fragments. GUS activities of each transformant were examined in (A) seed, (B) leaf, (C) stem and (D) root, individually. Constructs used in the study are shown in the Figure 1. Each dot represents the specific GUS activity of a single independently transformed tobacco plant. The number of the plants assayed per constructs is shown in the parenthesis. Bars indicate the average value of the specific activity of individual construct. Relative GUS activity to the control (-90Δ35S) is shown by the numbers to the above of each column in the figure (e.g., x2.6). Significant difference between the mean value from a given construct and the control is assayed by t-test and pointed out by single ($p < 0.05$) or double ($p < 0.1$) asterisks beside the relative GUS activity.

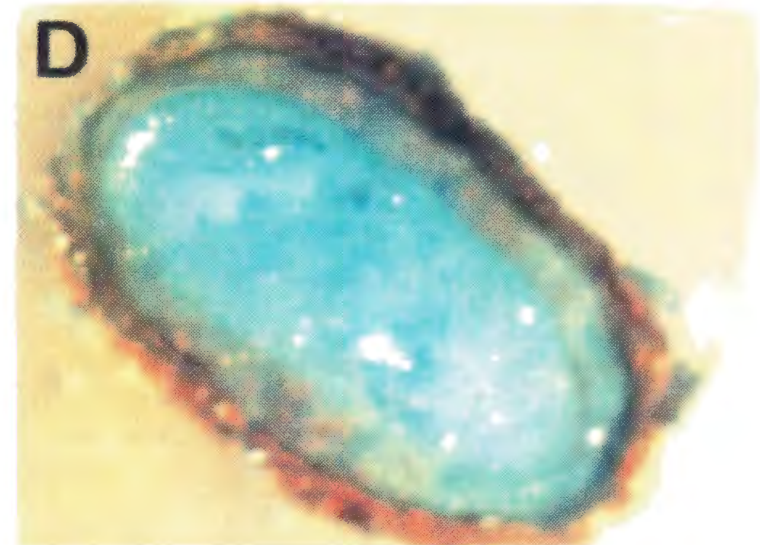
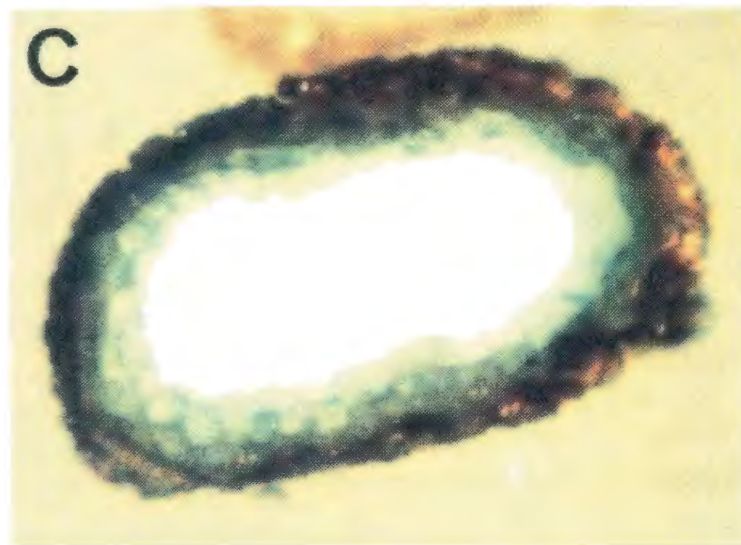
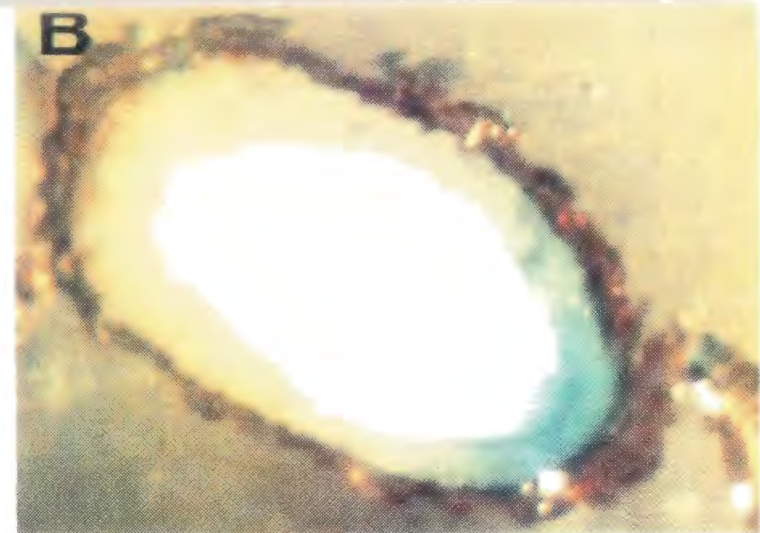
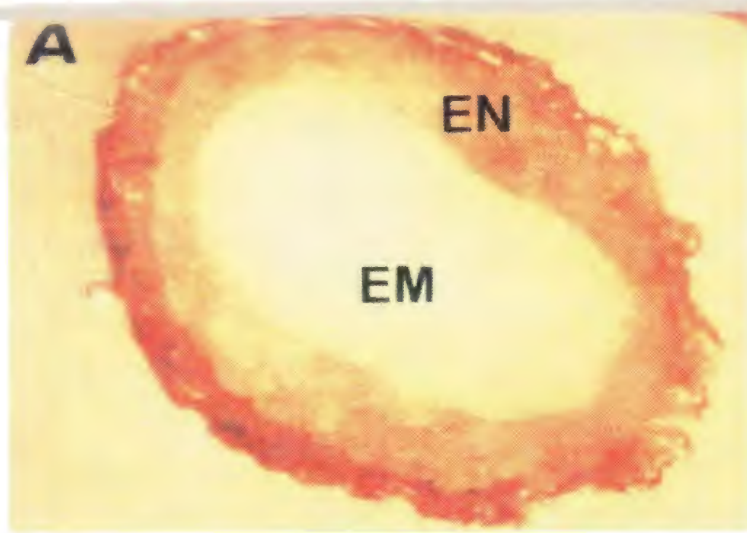


Figure IV-3. Histochemical GUS staining of transgenic tobacco seeds carrying the -90 Δ 35S with various *GluA-3* truncated fragments. A, Non-transformant; B, control (-90 Δ 35S); C, Nm construct; D, MAm construct; Em, embryo; En, endosperm.

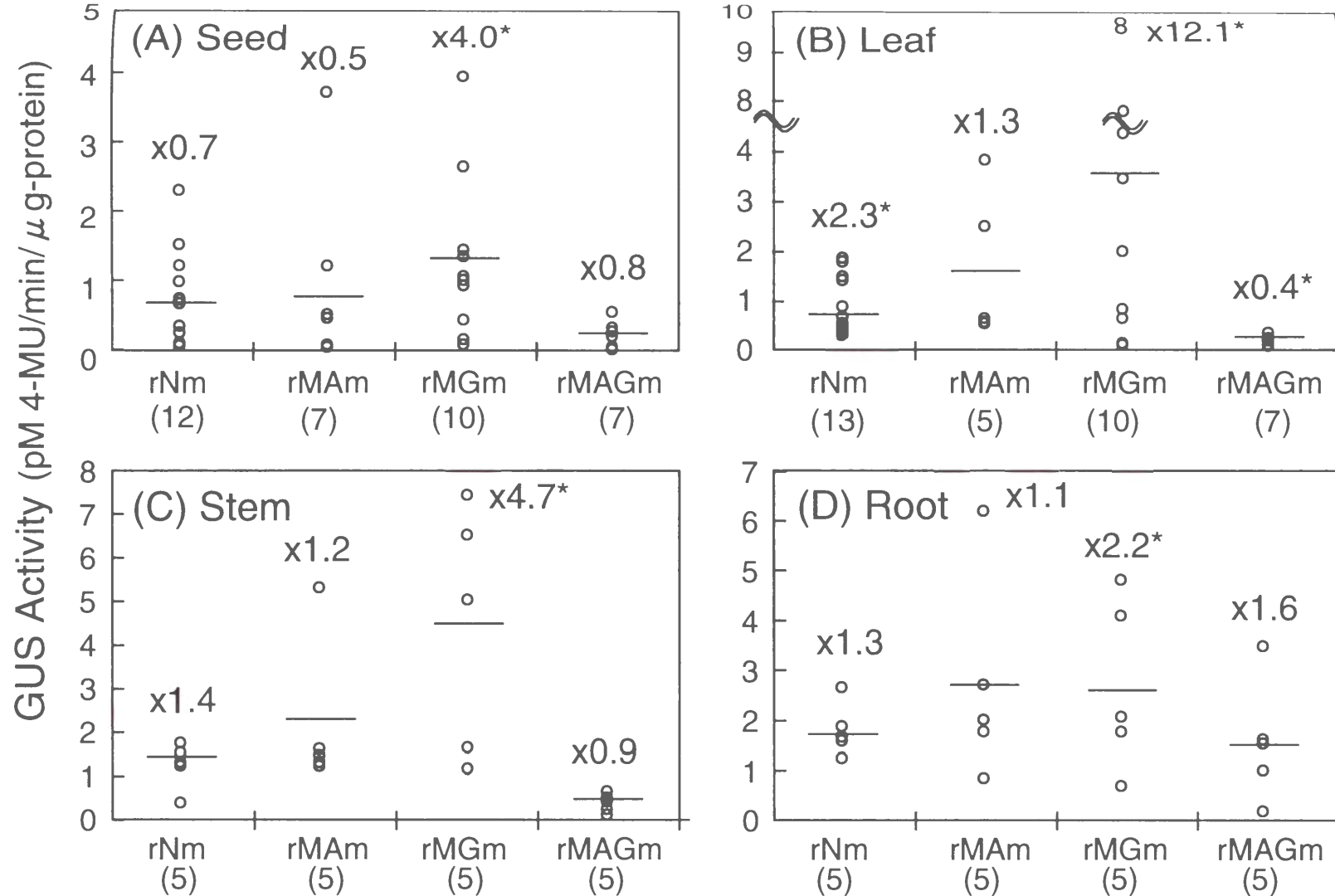


Figure II-4. Effect of the orientation of normal and mutated 45 bp fragments on GUS activity in (A) seed, (B) leaf, (C) stem and (D) root of each transformant. Constructs used in the study are shown in the Figure 1. Each dot represents the specific GUS activity of a single independently transformed tobacco plant. The number of the plants assayed per constructs is shown in the parenthesis. Bars indicate the average value of the specific activity of individual construct. Relative averaged GUS activity to the construct in the forward orientation (shown in Figure 2) is shown by the numbers to the above of each column in the figure (e.g., x4.0 = four folds). Significant difference between the mean value from a given construct and a construct in the forward orientation is assayed by t-test ($p < 0.05$) and pointed out by an asterisk beside the relative GUS activity.

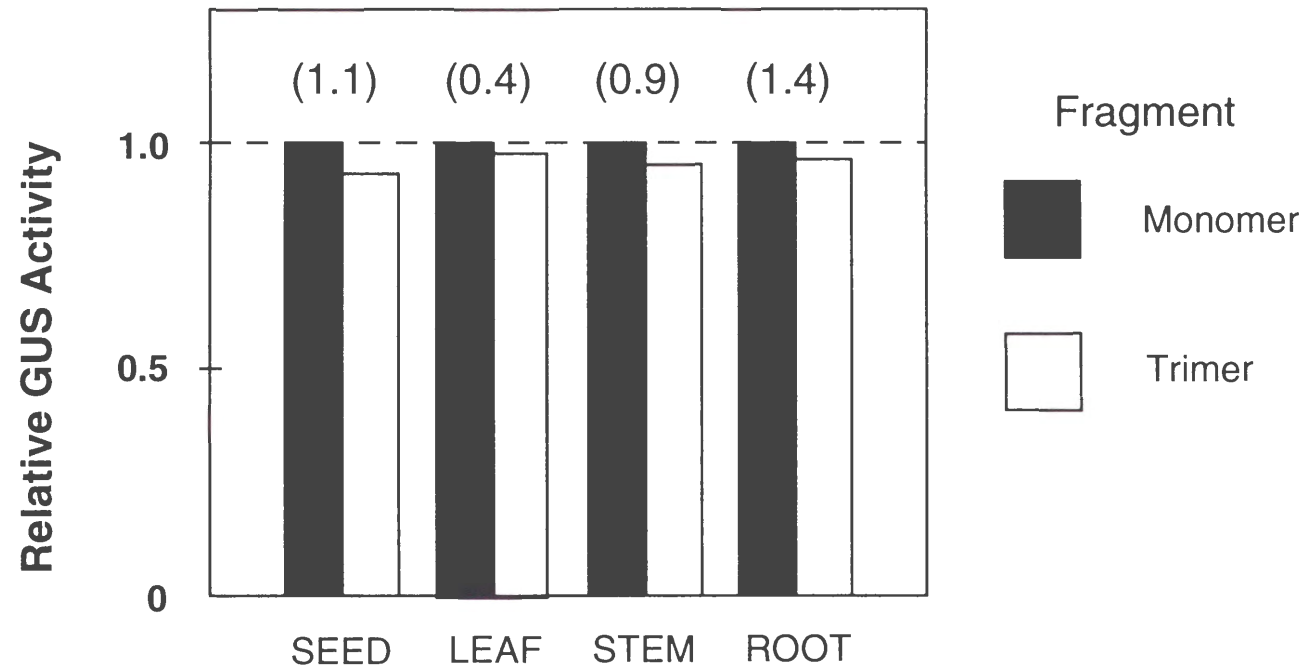


Figure IV-5. Relative averaged GUS activities of the *GluA-3* trimerized construct to the monomer construct in seed, leaf, stem and root. Each of the actual GUS activity (pmol 4MU/min/μg-protein) of the trimerized construct is shown in the parenthesis above the column.

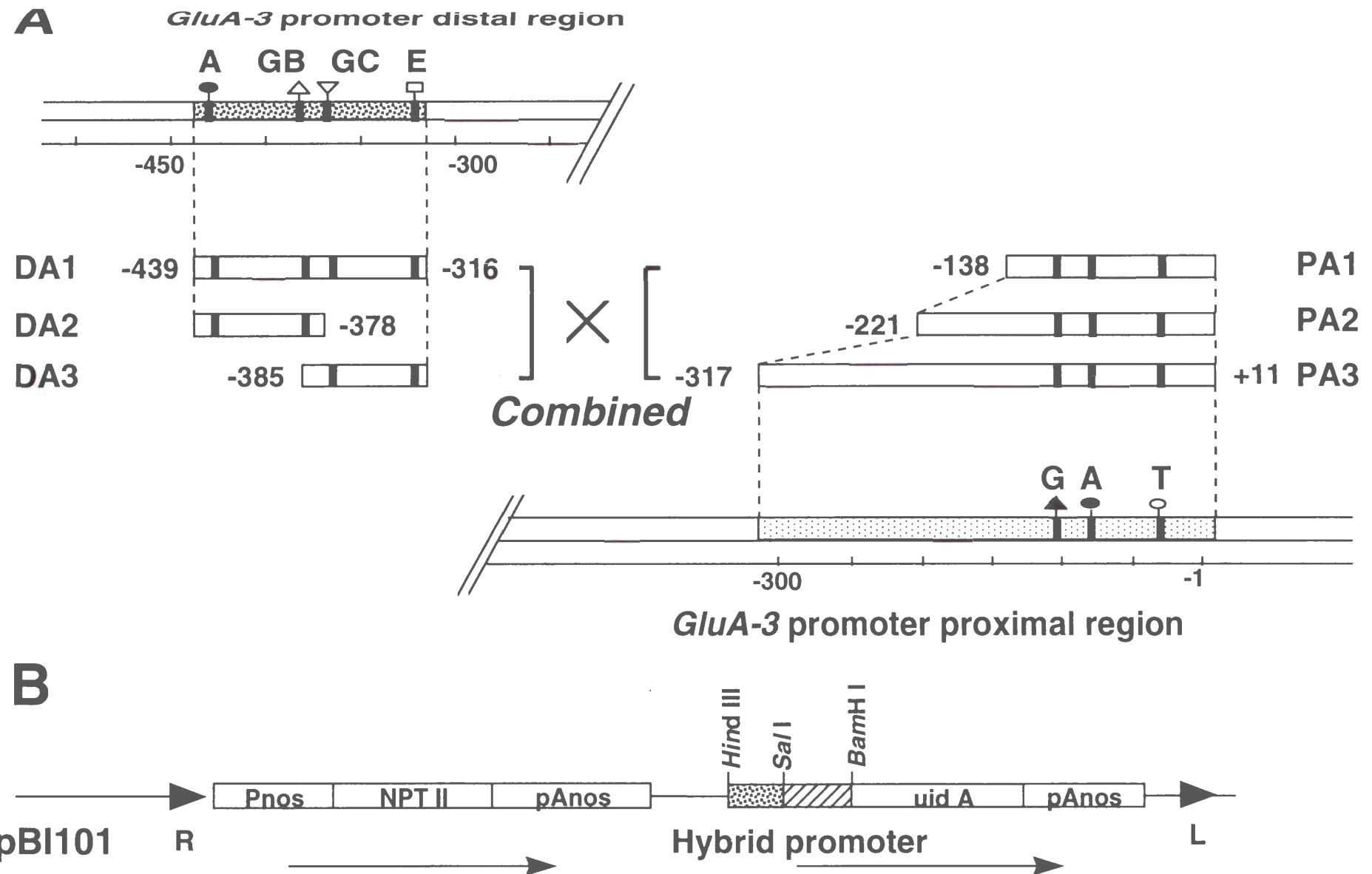


Figure V-1. Construction of the hybrid promoters consisted of *GluA-3* distal :: *GluA-3* proximal sequences. (A) Combinatorial aspects of the hybrid promoters. Three *GluA-3* distal sequences and three *GluA-3* proximal sequences were combined each other, and nine hybrid promoters were constructed in total. The motifs discussed in the text are indicated by boldface letters and vertical bars in the figure; A, AACA motif; E, endosperm motif; G, GCN4 motif; GB, G-box-like motif; GC, GCAA motif; T, TATA box. (B) Structure of binary vector. Hybrid promoters were cloned into the *Hind*III and *Bam*HI sites of pBI101.

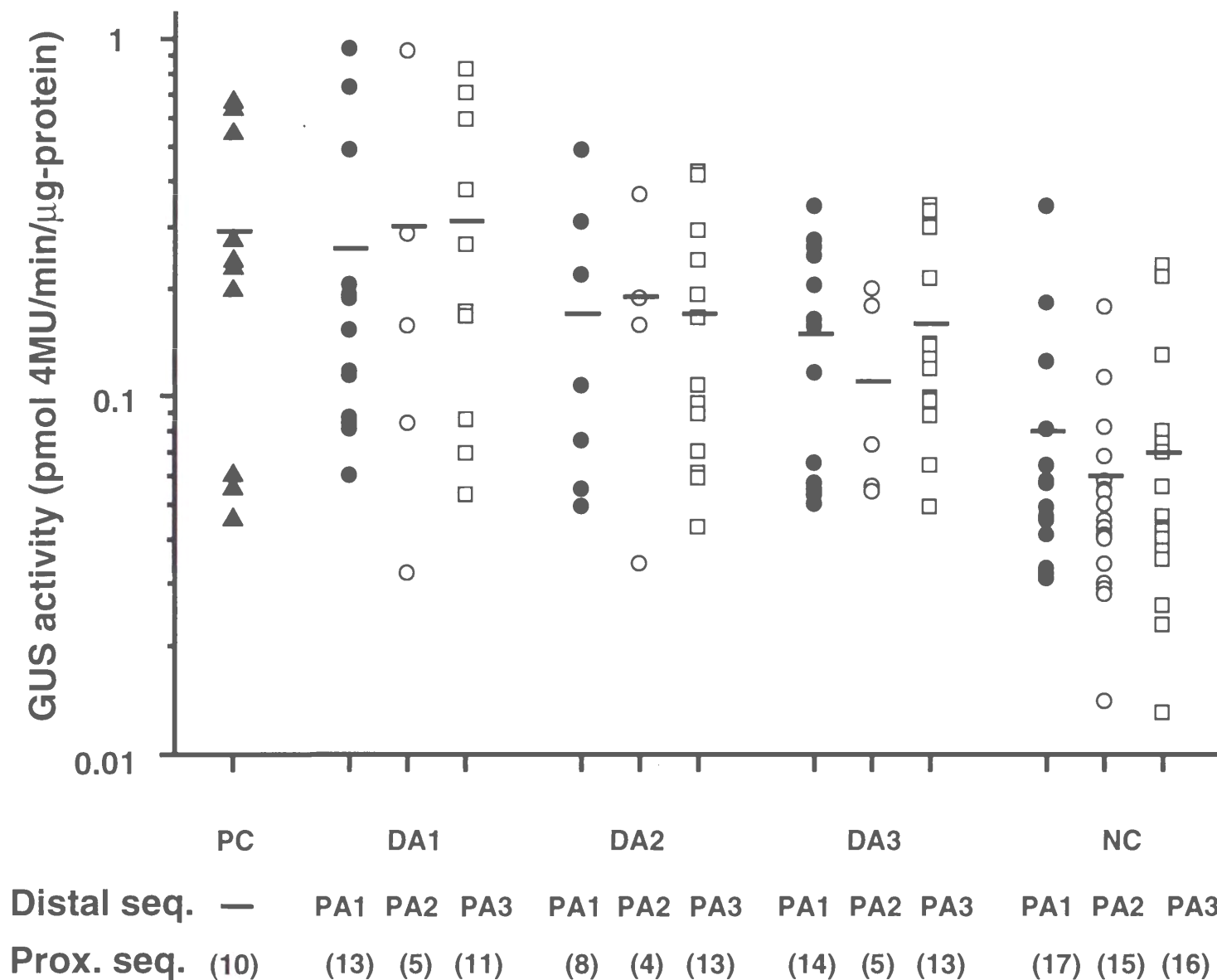


Figure V-2. GUS activity in transgenic tobacco seeds carrying the hybrid promoters consisting of the *GluA-3* distal :: *GluA-3* proximal sequences. Effect of each hybrid promoter is indicated as the combination of the distal and proximal sequences: closed triangle, the *GluA-3* native promoter between positions -437 and +11 (PC, positive control); closed circle, hybrid promoters using PA1 proximal sequence; open circle, hybrid promoters using PA2 proximal sequence; open square, hybrid promoters using PA3 proximal sequence. NC means the activities from constructs of each proximal sequences alone as negative control. Each dot represents the GUS activity for individual transgenic tobacco line. The number of plants tested is shown in parentheses. Bar indicates the average value of the GUS activity for individual construct. For comparing the effects of hybrid promoters, all transgenic plants carrying the *GluA-3* proximal regions were placed into five distinct groups concerning combinations with the distal region. Significant differences in expression between the two groups as determined by the two-sample *t*-test were observed only in combinations of; DA1 vs DA2 ($p < 0.10$, $n = 54$), DA1 vs DA3 ($p < 0.05$, $n = 61$), PC vs DA2 ($p < 0.10$, $n = 35$), NC vs DA3 ($p < 0.01$, $n = 42$), NC vs each of the others ($p < 0.01$ each, $n = 58, 77, 73, 80$).

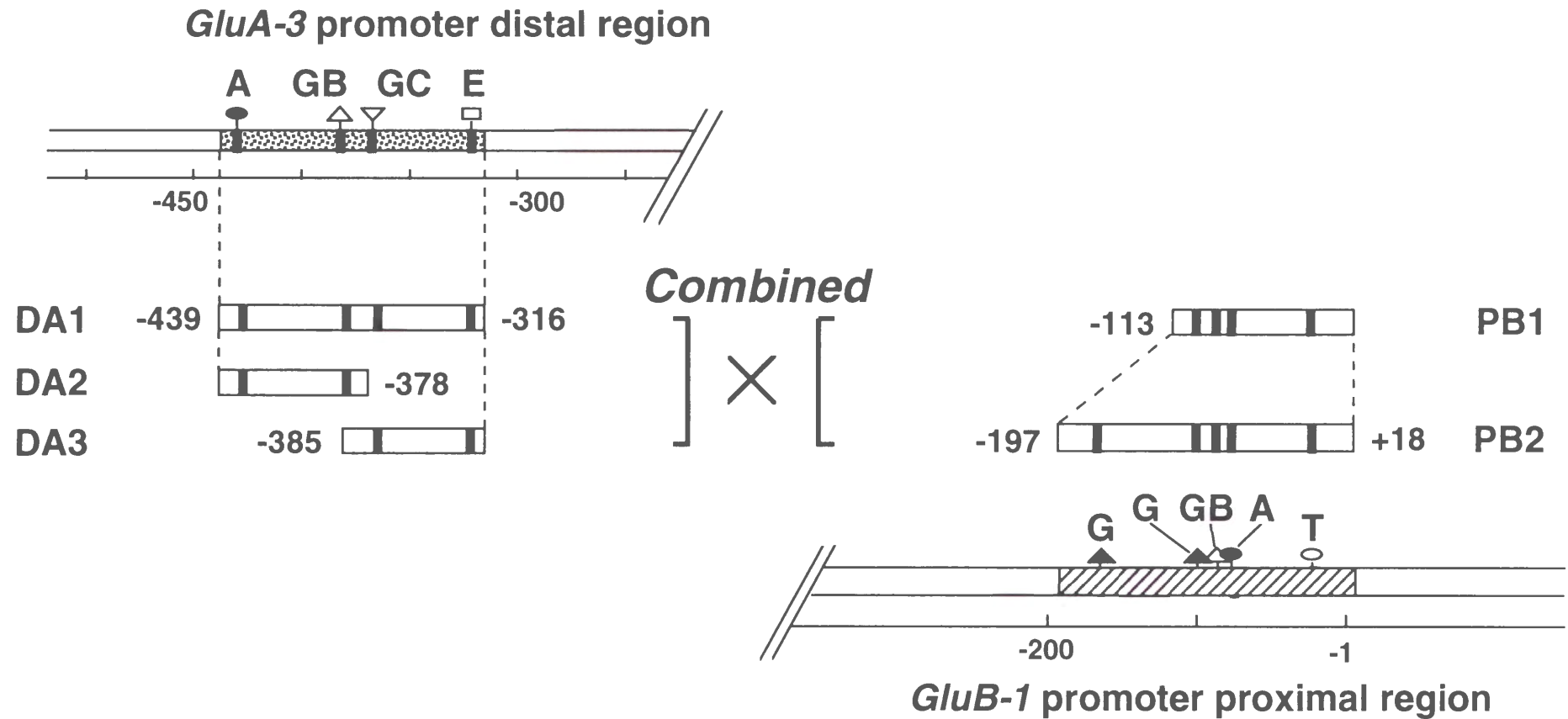


Figure V-3. Construction of the hybrid promoters consisted of *GluA-3* distal :: *GluB-1* proximal sequences. Three *GluA-3* distal sequences and two *GluA-3* proximal sequences were combined each other, and six hybrid promoters were constructed in total. The motifs discussed in the text are indicated by boldface letters and vertical bars in the figure; A, AACA motif; E, endosperm motif; G, GCN4 motif; GB, G-box-like motif; GC, GCAA motif; T, TATA box. These hybrid promoters were subcloned into the *Hind*III and *Bam*HI sites of pBI101 shown in Fig. V-1 (B).

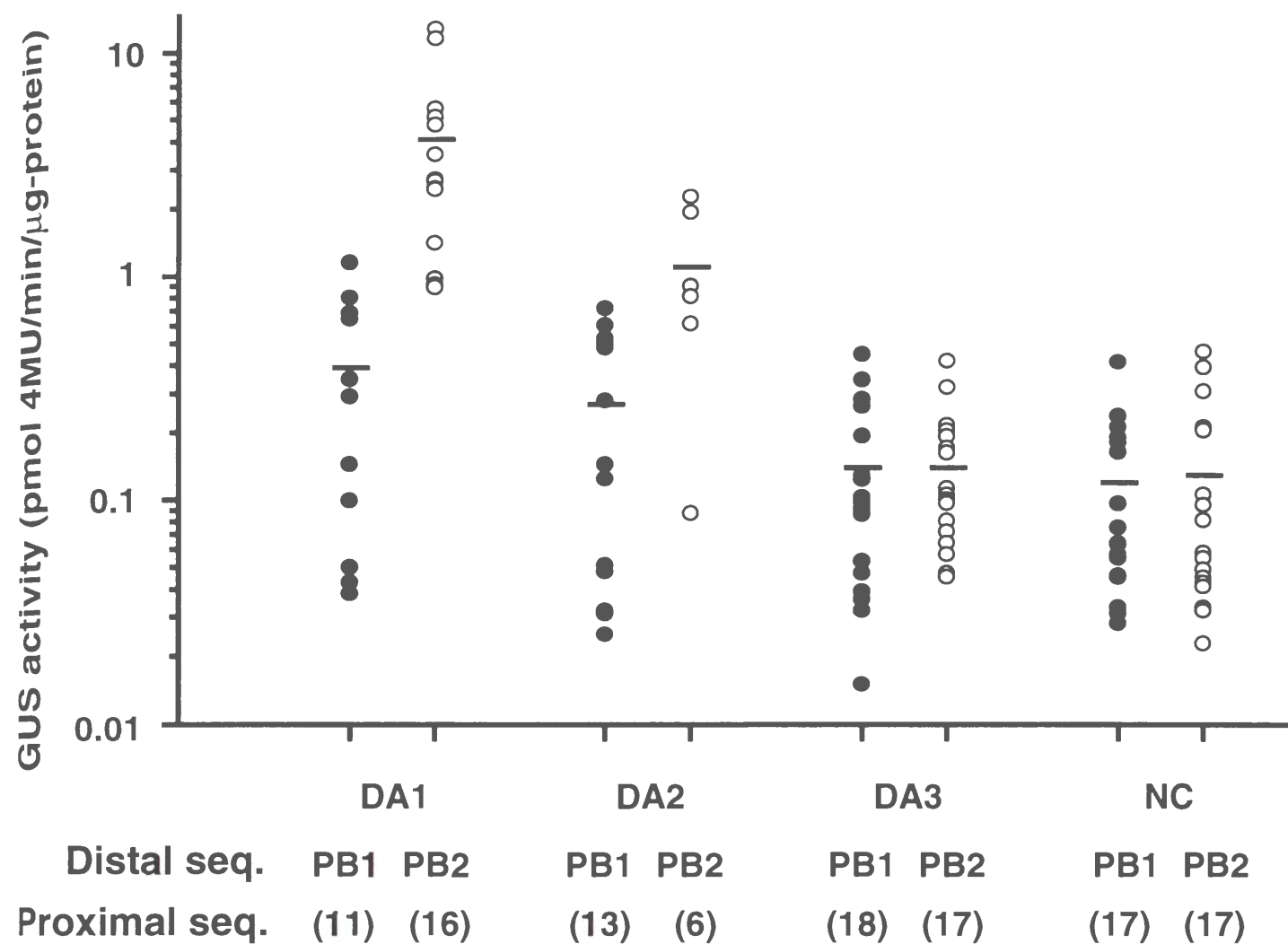


Figure V-4. GUS activity in transgenic tobacco seeds carrying hybrid promoters consisting of the *GluA-3* distal :: *GluB-1* proximal sequences. Closed circle, hybrid promoters using PB1 proximal sequence; open circle, hybrid promoters using PB2 proximal sequence. Each dot represents the GUS activity for each individual transgenic tobacco line. The number of the plants tested is shown in parentheses. Bar indicates the average value of the GUS activity for individual construct. For comparing the effects of hybrid promoters, all transgenic plants carrying the *GluB-1* proximal regions were placed into four distinct groups concerning combinations with the distal region. Significant differences in expression between the two groups as determined by the two-sample *t*-test were observed only in combinations of; DA1 vs DA2 ($p < 0.05$, $n = 46$), DA1 vs DA3 ($p < 0.01$, $n = 62$), DA2 vs DA3 ($p < 0.01$, $n = 54$), NC vs DA1 ($p < 0.01$, $n = 61$), NC vs DA2 ($p < 0.01$, $n = 53$).

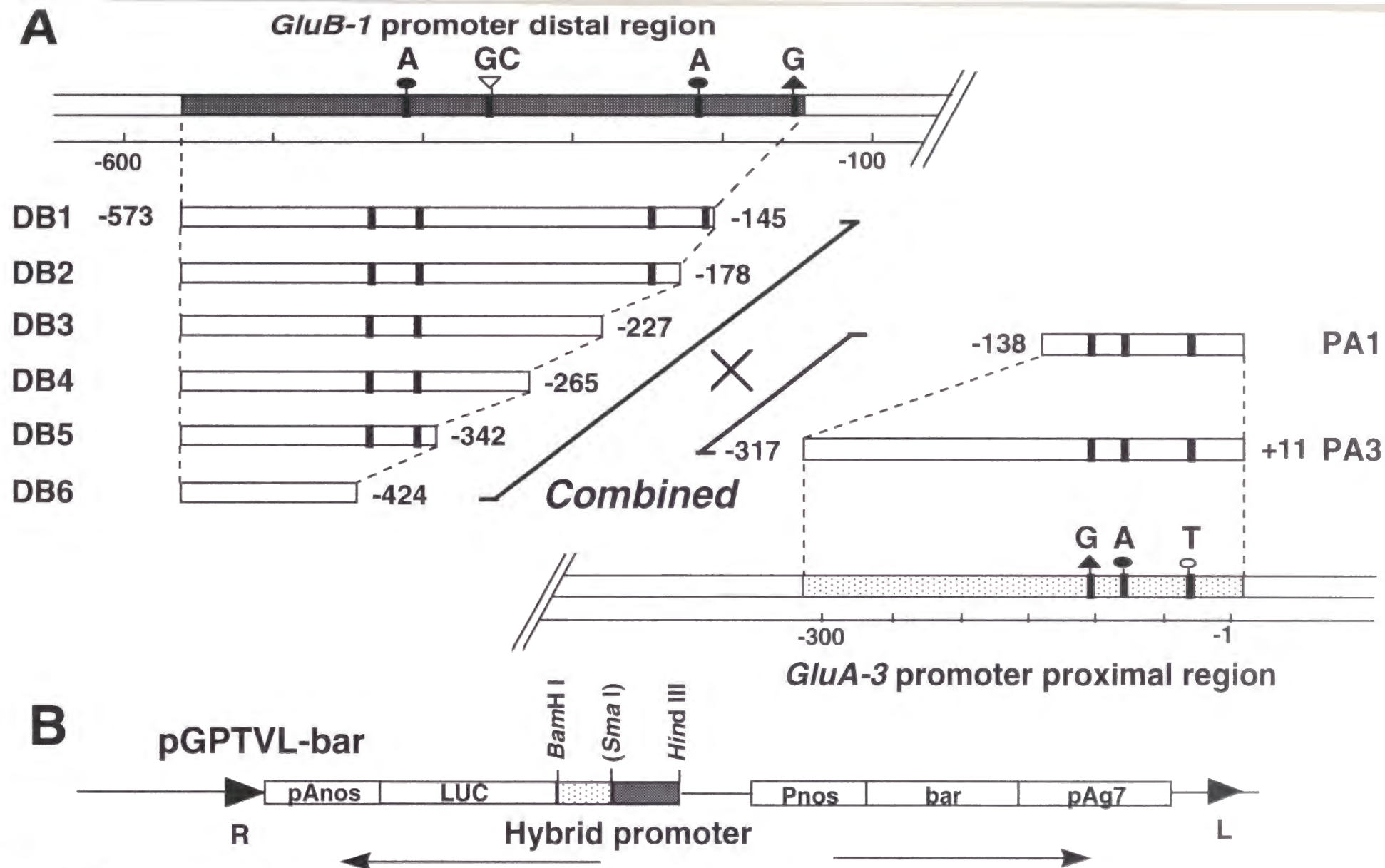


Figure V-5. Construction of the hybrid promoters consisted of *GluB-1* distal :: *GluA-3* proximal sequences.

(A) Combinatorial aspects of the hybrid promoters. Six *GluB-1* distal sequences and two *GluA-3* proximal sequences were combined each other, and twelve hybrid promoters were constructed in total. The motifs discussed in the text are indicated by boldface letters and vertical bars in the figure; A, AACA motif; G, GCN4 motif; GC, GCAA motif; T, TATA box. (B) Structure of binary vector. Hybrid promoters were cloned into the HindIII and BamHI sites of pGPTVL-bar.

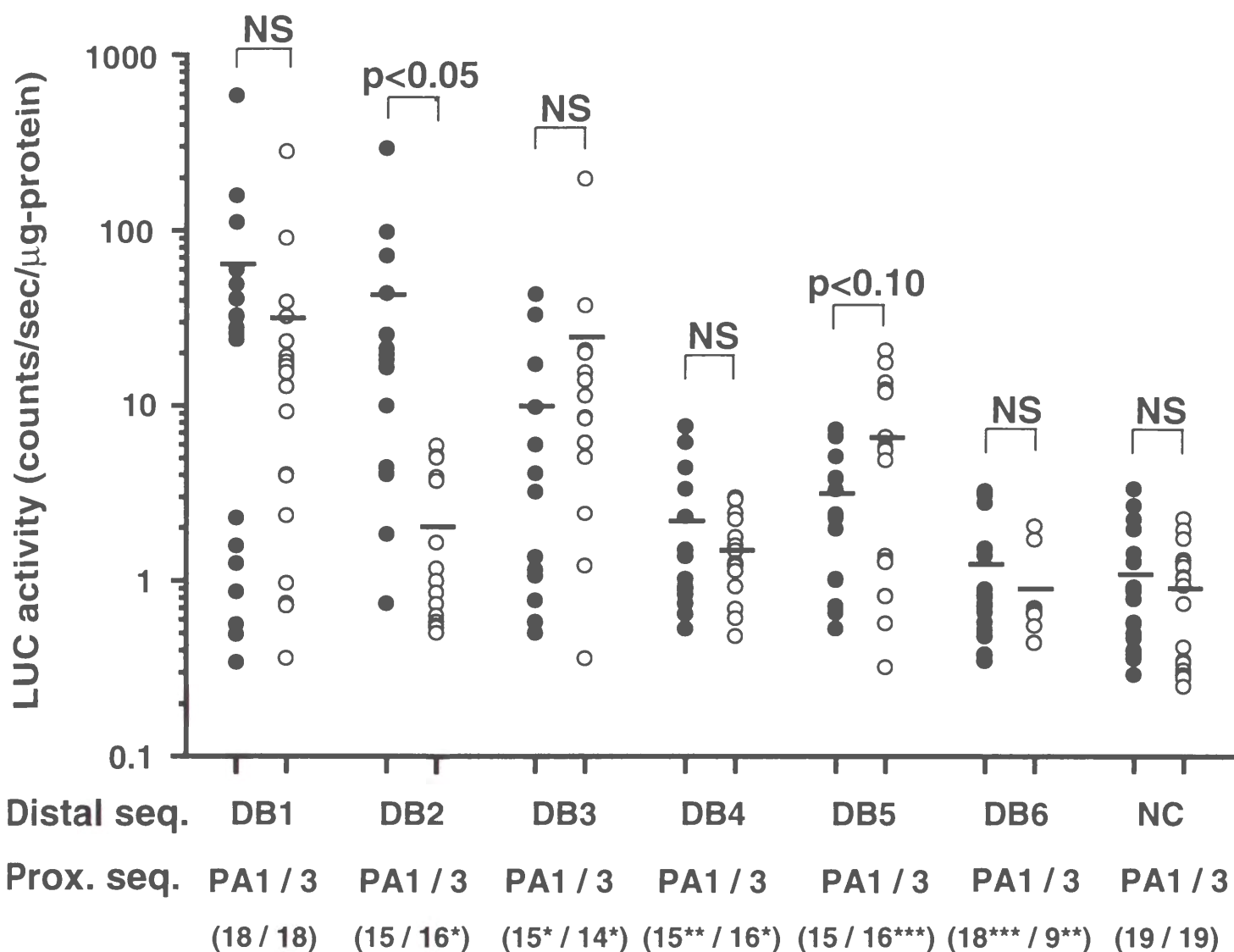


Figure V-6. LUC activity in transgenic tobacco seeds carrying the hybrid promoters consisted of the *GluB-1* distal :: *GluA-3* proximal sequences. Closed circle, hybrid promoters using PA1 proximal sequence; open circle, hybrid promoters using PA3 proximal sequence. Each dot represents the LUC activity for individual transgenic tobacco line. The number of the plants tested is shown in parentheses. Bar indicates the average value of the LUC activity for individual construct. Significant difference between the mean value from a given construct and the adjacent construct containing the same proximal and the longer distal sequence is assayed by two-sample *t*-test. It is pointed out by a single ($p < 0.10$), double ($p < 0.05$) and triple ($p < 0.01$) asterisk beside the number of the plants assayed. Significant difference between the mean values from the construct containing the same proximal sequence was also tested by two-sample *t*-test and the level of significance (e.g. $p < 0.05$ or NS, not significant) is indicated directly on the top of the dots.

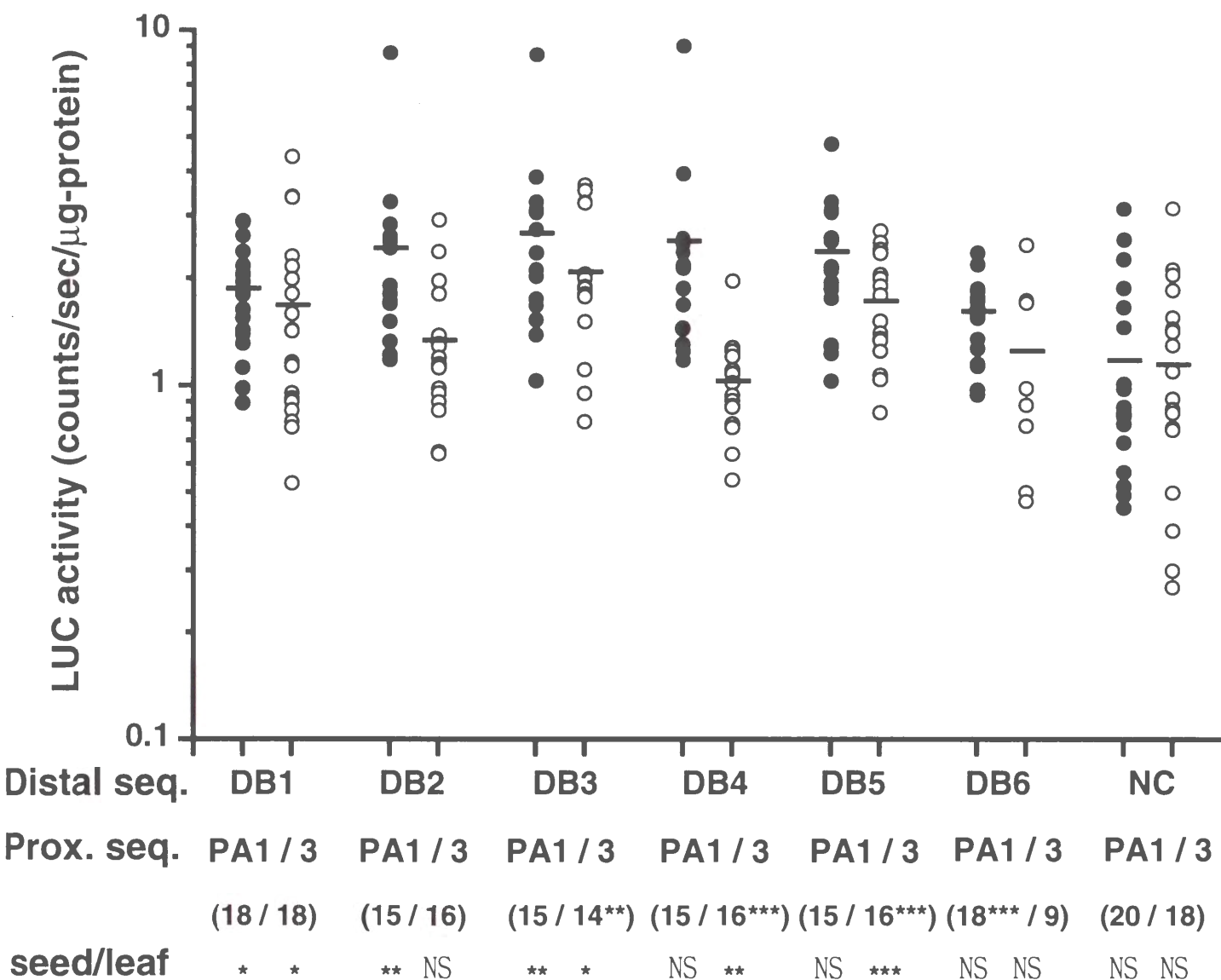


Figure V-7. LUC activity in transgenic tobacco leaves carrying the hybrid promoters consisted of the *GluB-1* distal :: *GluA-3* proximal sequences. Closed circle, hybrid promoters using PA1 proximal sequence; open circle, hybrid promoters using PA3 proximal sequence. Each dot represents the LUC activity for individual transgenic tobacco line. The number of the plants tested is shown in parentheses. Bar indicates the average value of the LUC activity for individual construct. Significant difference between the mean value from a given construct and the adjacent construct containing the same proximal and the longer distal sequence is assayed by two-sample *t*-test. It is pointed out by a single ($p < 0.10$), double ($p < 0.05$) and triple ($p < 0.01$) asterisk beside the number of the plants assayed. Significant difference between the mean values of seed and leaf containing the same construct was also tested by two-sample *t*-test and the level of significance (asterisks as listed above or NS, not significant) is indicated under the parentheses.

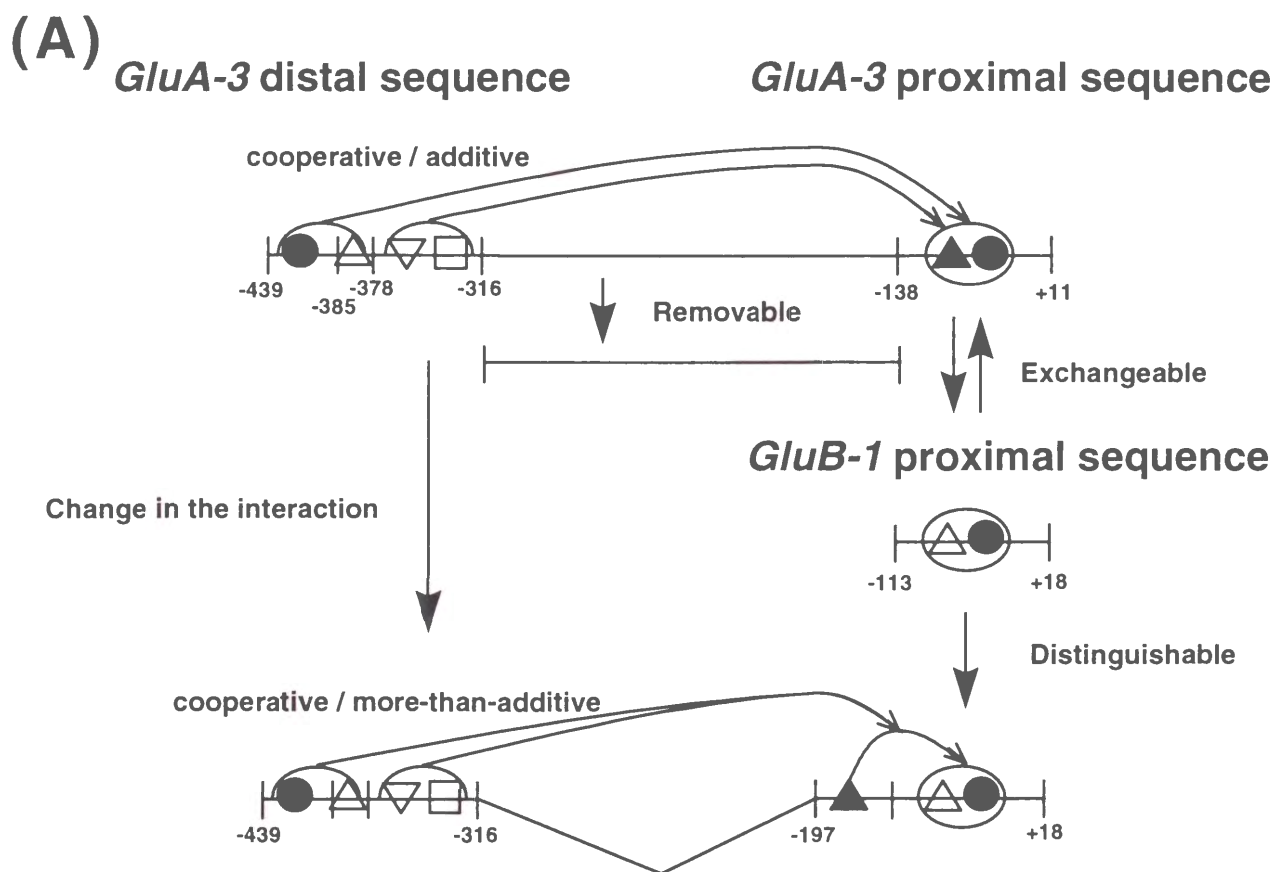


Figure V-8 (A). Schematic hypotheses for the interaction between the distal and proximal regions of the *GluA-3* and *GluB-1* genes.

Motifs discussed in the text are indicated by: closed circle, AACAA motif; open triangle in regular phase, G-box motif; open triangle in reversed phase, GCAA motif; open square, endosperm motif; closed triangle, GCN4 motif. Inseparable motifs from our knowledge are indicated in the half or whole ovals. The arrow with the narrow line indicates the putative interactions between the motifs.

(A) A model for the interaction between the *GluA-3* distal and *GluA-3* or *GluB-1* proximal sequences. Motifs in the *GluA-3* distal sequence may separately interact with motifs in the *GluA-3* proximal sequence. These motifs show an additive enhancing effect to the promoter activity. The proximal sequence of the *GluA-3* gene (-138/+11) could be exchangeable with that of *GluB-1* gene (-113/+18) and the sequence between positions -316 and -138 of the *GluA-3* gene may be removable without any change in the promoter activity. However, motifs in the *GluA-3* distal sequence synergistically interact with motifs in the longer *GluB-1* proximal sequence (-197/+18), and show a more-than-additive enhancing effect to the promoter activity.

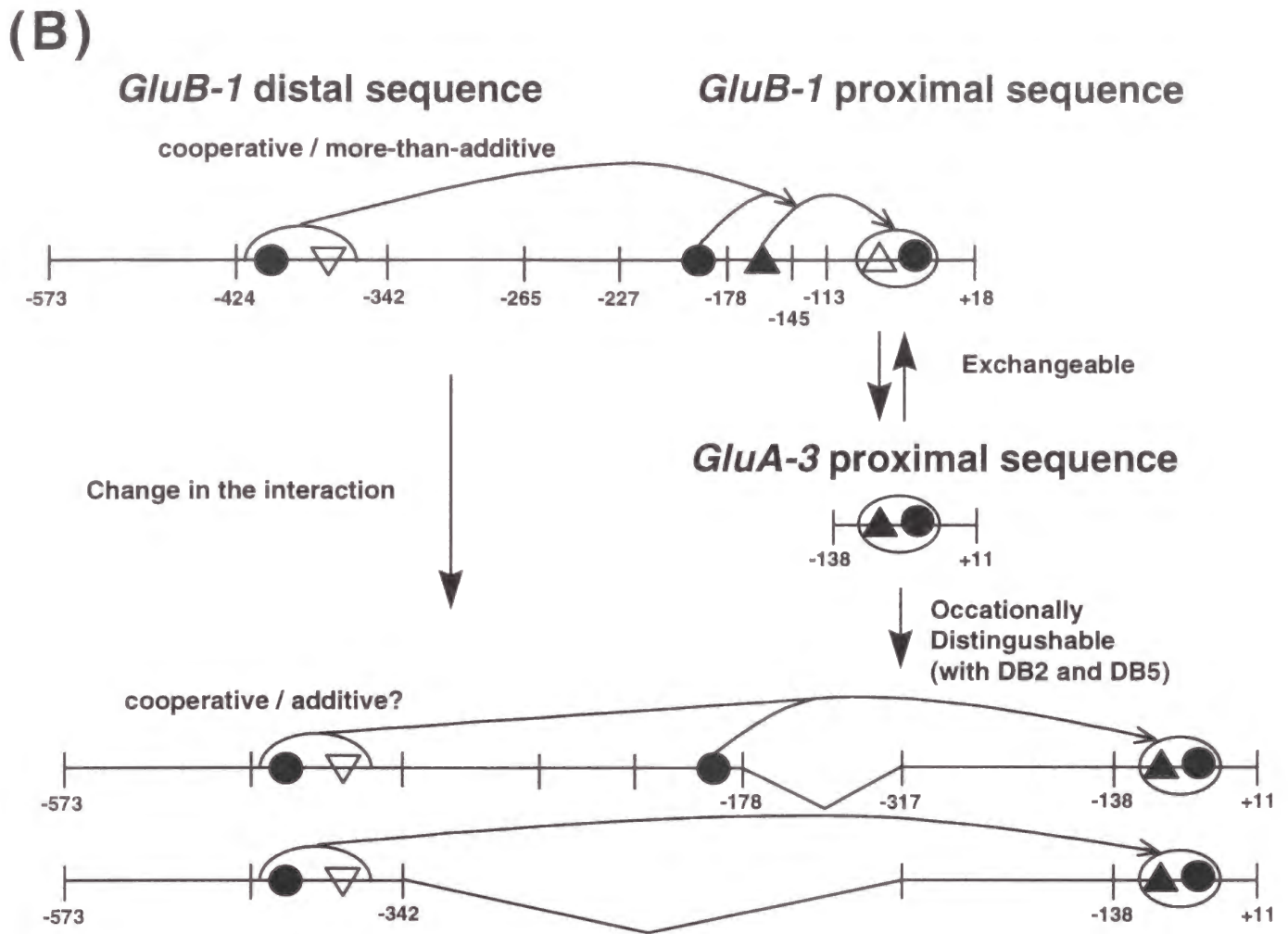


Figure V-8 (B). Schematic hypotheses for the interaction between the distal and proximal regions of the *GluA-3* and *GluB-1* genes.

(B) A model for the interaction between the *GluB-1* distal and *GluA-3* or *GluB-1* proximal sequences. Motifs in the *GluB-1* distal sequence may synergistically interact with motifs in the shorter *GluA-3* (-138/+11) and the native *GluB-1* proximal sequence. These motifs show a more-than-additive enhancing effect to the promoter activity. The proximal sequence of the *GluB-1* gene (-113/+18) could be exchangeable with that of the *GluA-3* gene (-138/+11) without any change in the promoter activity. However, these motifs may separately interact with motifs in the proximal sequence, when the *GluB-1* distal sequence are deleted to position -178 and more. This could have resulted in an additive enhancing effect to the promoter activity and occasionally distinguishable expression with the longer *GluA-3* proximal sequence (-317/+11).